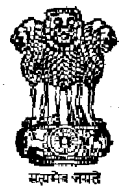
INDIAN PHARMACOPOEIA 2022

Volume I



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Ministry of Health & Family Welfare

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The Secretary-cum-Scientific Director
INDIAN PHARMACOPOEIA COMMISSION
Sector-23, Raj Nagar,
Ghaziabad-201 002, India
Tel: (91-120)–2783392, 2783400, 2783401
Website: www.ipc.gov.in
E.mail: lab.ipc@gov.in

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Notices

Legal Notices

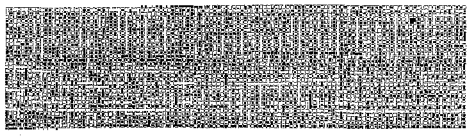
In India, under the Drugs and Cosmetics Act 1940, the current edition of Indian Pharmacopoeia is a book of standards for drugs included therein and the standards as included in the Indian Pharmacopoeia would be official. Also, in several other laws of India, the Indian Pharmacopoeia is recognised as the standard book. It is expedient that enquiry be made in each case in order to ensure that the provisions of any such law are being complied with. In general, the Drugs and Cosmetics Act, 1940, the Narcotic Drugs and Psychotropic Substances Act, 1985, the Poisons Act, 1919 and the rules framed thereunder should be consulted. These statutes empower the Government agencies to enforce the law using this compen-dium. The monographs of the Indian Pharmacopoeia should be read subject to the restriction imposed by those laws which are applicable.

If considered necessary, the standards included in Indian Pharmacopoeia can be amended and the Secretary-cum-Scientific Director is authorised to issue such amendments. Whenever such amendments are issued, the Indian Pharmacopoeia would be deemed to have been amended accordingly.

Patents and Trade Marks

In the Indian Pharmacopoeia, certain drugs and preparations have been included notwithstanding the existence of actual or potential rights in any part of the world. In so far as such substances are protected by Letters Patent their inclusion in the Indian Pharmacopoeia neither conveys, nor implies, licence to manufacture without due permission, authority, or licence from the person or persons in whom such rights exist.

The titles given under the individual monographs are public property. These titles cannot be patented as trade marks and no person is permitted to patent any trade mark devising the root of these titles.



Preface

The Ninth edition of the Indian Pharmacopoeia (IP 2022) is published by the Indian Pharmacopoeia Commission (IPC) on behalf of the Ministry of Health & Family Welfare, Government of India. The Indian Pharmacopoeia (IP) is published in fulfilment of the requirements of the Drugs and Cosmetics Act, 1940 and Rules thereunder. It prescribes the standards for drugs produced and/or marketed in India and thus contributes to the control and assurance of the quality of the medicines. The standards of the pharmacopoeia are authoritative and legally enforceable. It intends to help in the licensing of manufacturing, inspection and distribution of medicines. IP is published in continuing pursuit of the mission of IPC to improve the health of the people through ensuring the quality, safety and efficacy of medicines. The Commission has been receiving significant inputs from regulatory, industrial houses, academic institutions, national laboratories, individual scientists and others. Publication of IP at regular and shorter intervals is one of the main mandates of the Commission.

Indian Pharmacopoeia contains procedures for analysis and specifications for the determination of quality of pharmaceutical substances, excipients and dosage forms. IP monograph for an official substance or preparation includes the article's definition, description, identification, specific tests, assays, packaging, storage, labelling specifications and impurities profile, one or more analytical procedures for each test, acceptance criteria and other requirements.

The history of the IP began in the year 1833 when a Committee of the East India Company's Dispensary recommended the publication of a Pharmacopoeia and Bengal Pharmacopoeia and General Conspectus of Medicinal Plants were published in 1844, which mainly listed most of the commonly used indigenous remedies. This was followed by IP 1868, which covered both the drugs of British Pharmacopoeia (BP) 1867 and indigenous drugs used in India, with a Supplement published in 1869 incorporating the vernacular names of indigenous drugs and plants. However, from 1885 the BP was made official in India. A Drugs Enquiry Committee appointed in 1927 by the Government recommended the publication of a National Pharmacopoeia.

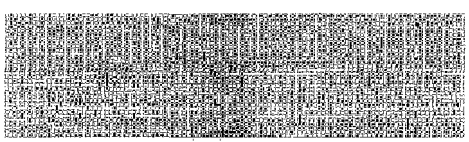
After independence, the Indian Pharmacopoeia Committee was constituted in 1948 for publication of IP as its main function, which published the first edition of IP in 1955, followed by a Supplement in 1960. This Pharmacopoeia contained both western and traditional system of drugs commonly used in India and the same policy continued while preparing the second edition of Indian Pharmacopoeia in 1966 and its Supplement 1975. There had been a phenomenal growth and development of the Indian Pharma industry since independence, especially from early 1970, both in the range of Active Pharmaceutical Ingredients (APIs) and the dosage forms produced. This totally transformed the profile of the Indian Pharmaceuticals market and Indian Pharma industry emerged as one of the important global suppliers of pharmaceutical products, both to the developed and developing countries. These developments posed major challenges for the IP to reflect the quality standards of the marketed drugs, which the subsequent editions and supplement of IP tried to address.

In view of these rapid advances, it was decided to publish a new edition of the Pharmacopoeia and its Addenda at regular and shorter intervals for which the Indian Pharmacopoeia Committee was reconstituted in 1978. Third edition of Indian Pharmacopoeia in 1985, its Addenda 1989 and 1991 respectively, inclusion of traditional system of drugs was limited. However, most of the new drugs manufactured and/or marketed were included, while only those herbal drugs which had definitive quality control standards had got place in it. In view of the continuing rapid increase in the range of drugs produced in India, the forth edition of IP 1996, its Addendum 2000, Supplement 2000 for Veterinary Products and Addendum 2002 were published. The Addendum 2005 was published by the IPC which included a large number of antiretroviral drugs and raw plants commonly used in making medicinal products not covered by any other pharmacopoeias, which attracted much global attention. The IP Committee decided to delete the obsolete or less used product monographs and added monographs based on the therapeutic merit, medical need and extent of use of such articles in the country.

The Indian Pharmacopoeia Commission was established in year 2005. It provided systematic approach and practices for publication of fifth edition of IP 2007 containing 271 new monographs with focus on those drugs and formulations that cover the National Health Care Programmes and the National Essential Medicines and Addendum 2008 to the IP 2007 containing 72 new monographs.

The sixth edition of IP 2010 contained monographs on antiretroviral, anticancer, antituberculosis and herbal drugs. It further emphasized on biological monographs such as Vaccines, Immunosera for human use, Blood products, Biotechnological and Veterinary (Biological and non-biological) preparations. Addendum 2012 to the IP 2010 was published which had taken care of the Amendments to IP 2010 and also incorporated 52 new monographs.

The seventh edition of Indian Pharmacopoeia was published in the year 2014 which incorporated about 577 monographs consisting of APIs, excipients, dosage forms, Radiopharmaceuticals, herbal and veterinary products.



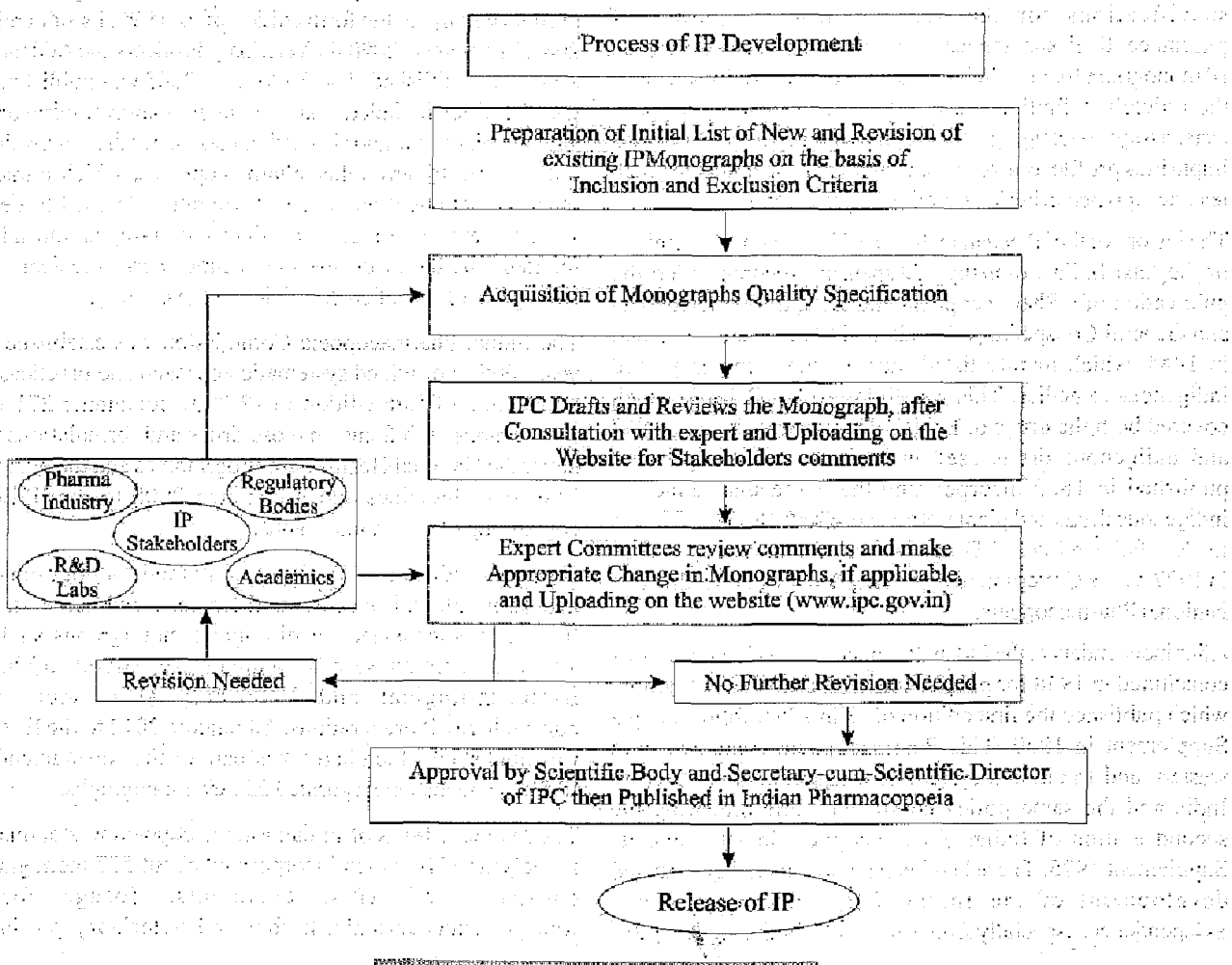
Addendum 2015 and Addendum 2016 to the IP 2014 were published which had taken care of the Amendments to IP 2014 and also incorporated 82 new monographs in Addendum 2015 and 89 new monographs in Addendum 2016 respectively.

The eighth edition of Indian Pharmacopoeia was published in year 2018 which incorporated 270 new mongoraph of API, Excipients, Dosage forms, Herbs & Herbal Products, Vaccines and Immunosera for Human Use, Blood and Blood Related Products, Biotechnology Derived Therapeutic Products and Radiopharmaceutical Preparation. Addendum 2019 and Addendum 2021 to IP 2018 were published which had taken care of amendmnets to IP 2018 and also incorporated 66 monographs in both Addendum 2019 and Addendum 2021.

The ninth edition of Indian Pharmacopoeia (IP- 2022) is published in accordance with the principles and designed plan decided by the Scientific Body of the IPC. To establish transparency in setting standards for this edition, the contents of new monographs, revised appendices and other information have been publicized on the website of the IPC, besides following conventional approach of obtaining comments. The feedback and inputs have been reviewed by the relevant Expert Committee to ensure the feasibility and practicability of the standards and methods revised. The principle of "openness, justice and fairness" is kept in mind during compiling and editing the contents of this edition.

Public Review and Comment Process for standards development related to this edition of the Indian Pharmacopoeia has given special attention to incorporate comments from stakeholders. The methodology adopted is appended below:

The IPC Secretariat and Indian Pharmacopoeia Laboratory (IPL) staff, with the support of different advisory Expert Committee and Expert Members of the Scientific Body have examined the suitability of the standards. In order to make IP 2022 user friendly, the existing formatting pattern has been suitably revised. The standards prescribed in this edition are encouraged to adhere with the concept of harmonization, keeping in view the technological status for manufacture and analysis of drugs and pharmaceuticals in the country without compromising with the quality of the products. It strives to


Process of IP Development
Preparation of Initial List of New and Revision of existing IPMonographs on the basis of Inclusion and Exclusion Criteria
Acquisition of Monographs Quality Specification
IPC Drafts and Reviews the Monograph, after Consultation with expert and Uploading on the Website for Stakeholders comments
Pharma Industry
Regulatory Bodies
IP Stakeholders
R&D Labs
Academics
Expert Committees review comments and make Appropriate Change in Monographs, if applicable, and Uploading on the website (www.ipc.gov.in)
Revision Needed
No Further Revision Needed
Approval by Scientific Body and Secretary-cum-Scientific Director of IPC then Published in Indian Pharmacopoeia
Release of IP

update the existing monographs as well as incorporating the new monographs of drug substances based on clinical use of medicines in India and improving their test protocols. The IP 2022 has been considerably revised and improved in respect of the requirements of monographs, general chapters, monographs of dosage form and testing protocols by introducing advanced technology. The contents of general chapters and monographs of dosage forms are by and large revised in consonance with those adopted internationally. The monographs of combined dosage forms widely marketed in India have been given special attention.

In addition, emphasis has been put to bring out harmonisation in Appendices to establish a sound connection between individual monographs and the relevant appendices, so as to make this edition precise and well structured. Number of monographs and appendices are expanded further to incorporate the latest technological advancement and regulatory compliance. Constant efforts have been made to unify the National Drug Standards and to bring them in line with the International Standards progressively by addition of monographs of fixed dose combination drugs and adopting current methodology and constantly advancing technological standards.

IPC provides the official Indian Pharmacopoeia Reference Substances (IPRS) as primary reference standards and impurity standards to the stakeholders as per the requirements of monographs and the list of available IPRS and impurity standards is updated from time to time on the official website of IPC (www.ipc.gov.in).

This is the ninth edition of the Indian Pharmacopoeia. It comprises of four volumes. Each volume has got different features.

Volume I: Notices; Preface; About Indian Pharmacopoeia Commission; Acknowledgements; Introduction; General Notices; General Chapters; Reference Data and Index.

Volume II: General Notices; General Monographs on Active Pharmaceutical Ingredients and Dosage Forms; Drug Substances, Dosage Forms and Pharmaceutical Aids (A to M).

Volume III: General Notices; Drug Substances, Dosage Forms and Pharmaceutical Aids (N to Z); Vitamin, Minerals, Amino Acids, Fatty Acids etc.; Phytopharmaceuticals; Herbs and Herbal Products; Vaccines and Immunosera for Human Use; Blood and Blood-related Products; Biotechnology Derived Therapeutic Products; Allergen Products and Radiopharmaceutical Preparations.

Volume IV: General Notices; Notices; Veterinary Products and Index.

The Standards prescribed in the Indian Pharmacopoeia are to establish the compliance with regulatory requirements on an article. The criteria to be adhered to are:

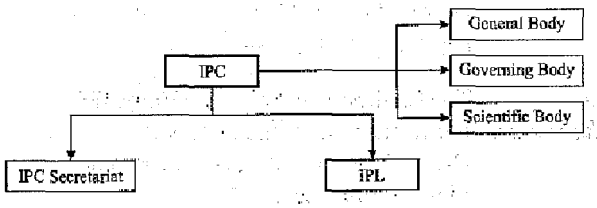
(i) The interpretation of a monograph must be in accordance with all the general requirements, testing methods, texts and notices pertaining to it, in the IP.

(ii) A product is not of standard quality unless it complies with all the requirements of the monograph.

The IP 2022 incorporates 222 monographs inclusive of 66 monographs of IP Addendum 2019, 66 monographs of IP Addendum 2021 and newly added 92 monographs of API's, Excipients, Dosage Forms; Vitamin, Minerals, Amino Acids, Fatty Acids etc.; Herbs and Herbal Products; Vaccines and Immunosera for Human Use; Blood and Blood-related Products; Biotechnology Derived Therapeutic Products. It is intended that this edition would play a significant role in improving the quality of medicines which in turn will promote public health and accelerate the growth and development of pharma sector.

Indian Pharmacopoeia Commission

The Indian Pharmacopoeia Commission (IPC) has been formed as an autonomous institution under the Ministry of Health and Family Welfare's Resolution bearing dated 6th May, 2008 published in the Official Gazette of the Government of India. It is a three-tier structure comprising of the General Body of 25 members, Governing Body of 14 members and Scientific Body of 15-23 members from different related scientific fields. The Secretary, Ministry of Health and Family Welfare, is the Chairperson and the Chairperson-Scientific Body is the Co-Chairperson of the Commission. The Secretary-cum-Scientific Director is the Chief Scientific and Executive Officer of the Commission.



IPC-Structure

The primary responsibility of the Scientific Body is to provide guidelines for standards development related to the Indian Pharmacopoeia with the assistance of its Expert Working Groups.

The Indian Pharmacopoeia is published in continuing pursuit of the Mission, Vision and Objectives of the IPC.

Mission

To promote public and animal health in India by bringing out authoritative and officially accepted standards for quality of drugs including active pharmaceutical ingredients, dosage forms and excipients used by health professionals, patients and consumers.

Vision

To promote the highest standards of drugs for use in human and animals within practical limits of the technologies available for manufacture and analysis.

Objectives

- To develop comprehensive monographs for drugs to be included in the Indian Pharmacopoeia, including active pharmaceutical ingredients, dosage forms and pharmaceutical aids as well as medical devices and to keep them updated by revision on a regular basis.
- To develop monographs for herbal drugs, both raw drugs and extracts/formulations therefrom.
- To accord priority to monographs of drugs included in the National List of Essential Medicines and their dosage forms.
- To take note of the different levels of sophistication in analytical testing/ instrumentation available while framing the monograph.
- To accelerate the process of preparation, certification and distribution of IP Reference Substances, including the related substances, impurities and degradation products.
- To collaborate with Pharmacopoeias like the Ph Eur, BP, USP, JP and International Pharmacopoeia with a view to harmonize with global standards.
- To review existing monographs periodically with a view to delete obsolete ones and amending those requiring upgradation/revision.
- To organize educational programs and research activities for spreading and establishing awareness on the need and scope of quality standards for drugs and related articles /materials.
- To publish the National Formulary of India for updating medical practitioners and health professionals.
- To act as a National Coordination Centre for Pharmacovigilance Programme of India.

The Governing Body

The composition of the Governing Body is given below:

Chairperson	The Secretary (Health & Family Welfare)
(*ex-officio*)	Ministry of Health & Family Welfare Government of India Nirman Bhawan New Delhi-110 011. (Shri Rajesh Bhushan)
Co-Chairperson	The Chairperson, Scientific Body, IPC Former Director General, ICMR C-191, 9th Floor, City View Apartment Plot No. 199, Sector-35 Noida-201 301. (Prof. N. K. Ganguly)
Member (*ex-officio*)	The Director General (DGHS) Ministry of Health & Family Welfare Nirman Bhawan New Delhi-110 011. (Prof. Sunil Kumar)
Member (*ex-officio*)	The Additional Secretary & Financial Advisor Ministry of Health & Family Welfare Nirman Bhawan New Delhi-110 011. (Shri Ashish Srivastava)

Member (*ex-officio*)
The Additional Secretary (Drugs)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Smt. Roli Singh)

Member (*ex-officio*)
The Joint Secretary (Regulations)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Dr. Mandeep Kumar Bhandari)

Member (*ex-officio*)
The Joint Secretary
Department of Pharmaceuticals
Ministry of Chemicals and Fertilizers
Shastri Bhawan
New Delhi-110 011.
(Shri N. Yuvaraj)

Member (*ex-officio*)
The Director (Drugs)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Shri Rajiv Wadhawan)

Member (*ex-officio*)
The Drugs Controller General (I)
Directorate General of Health Services
Ministry of Health & Family Welfare
FDA Bhawan, Kotla Road
New Delhi-110 002.
(Dr. V. G Somani)

Member (*ex-officio*)
The Director
National Institute of Biologicals
A-32, Sector-62, Institutional Area
Noida-201 309.
(Dr. Anup Anvikar)

Member
The President
Pharmacy Council of India
NBCC Centre, 3rd Floor
Plot No.2, Community Centre,
Maa Anandamai Marg,
Okhla Phase - I
New. Delhi - 110 020.
(Prof. Pramod Yeole)

Member
Dr. Pankaj Patel
C&MD
Zydus Cadila Healthcare Ltd.
Zydus Tower, 6th Floor
Opp. ISKCON Temple, Satellite Cross Roads
Sarkhej-Gandhinagar Highway
Ahmedabad-380 015

Member-Secretary (*ex-officio*)
The Secretary-cum-Scientific Director
Indian Pharmacopoeia Commission
Sector-23, Raj Nagar
Ghaziabad-201 002.
(Dr. Rajeev Singh Raghuvanshi)

The General Body

The composition of the General Body is as follows:

Chairperson (*ex-officio*)
The Secretary (Health & Family Welfare)
Ministry of Health & Family Welfare
Government of India
Nirman Bhawan
New Delhi-110 011.
(Shri Rajesh Bhushan)

Co-Chairperson
The Chairperson, Scientific Body, IPC
Former Director General, ICMR
C-191, 9th Floor, City View Apartment
Plot No. 199, Sector-35
Noida-201 301.
(Prof. N. K. Ganguly)

Member (*ex-officio*)
The Director General (DGHS)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Prof. Sunil Kumar)

Member (*ex-officio*)
The Additional Secretary & Financial Advisor
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Shri Ashish Srivastava)

Member (*ex-officio*)
The Additional Secretary (Drugs)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Smt. Roli Singh)

Member (*ex-officio*)
The Joint Secretary (Regulations)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Dr. Mandeep Kumar Bhandari)

Member (*ex-officio*)
The Joint Secretary
Department of Pharmaceuticals
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Shastri Bhawan
New Delhi-110 011.
(Shri N. Yuvaraj)

Member (*ex-officio*) The Director (Drugs)
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi-110 011.
(Shri Rajiv Wadhawan)

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Directorate General of Health Services
Ministry of Health & Family Welfare
FDA Bhawan, Kotla Road
New Delhi-110 002.
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Plot No.2, Community Centre,
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New. Delhi - 110 020.
(Prof. Pramod Yeole)

Member Dr. Pankaj Patel
C&MD
Zydus Cadila Healthcare Ltd.
Zydus Tower, 6th Floor
Opp. ISKCON Temple, Satellite Cross Roads
Sarkhej-Gandhinagar Highway
Ahmedabad-380 015

Member (*ex-officio*) The Director
Central Drugs Laboratory
3, Kyd Street
Kolkata-700 016.
(Shri C. Hariharan)

Member (*ex-officio*) From Regulatory Bodies
Central Drugs Standard Control Organisation
Directorate General of Health Services
FDA Bhawan, Kotla Road,
New Delhi-110 002.

Member (*ex-officio*) The Director
National Institute of Pharmaceutical Education and Research (NIPER)
Sector 67, SAS Nagar
Mohali-160 062.
(Prof. Dulal Panda)

Member (*ex-officio*) Commissioners in-charge of Drug Control Administration, Andhra Pradesh

Member (*ex-officio*) Commissioners in-charge of Drug Control Administration, Gujarat

Member (*ex-officio*) Commissioners in-charge of Drug Control Administration, Himachal Pradesh

Member (*ex-officio*) Commissioners in-charge of Drug Control Administration, Sikkim

Member (*ex-officio*) Commissioners in-charge of Drug Control Administration, Uttar Pradesh

Member (*ex-officio*) The Director
Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER)
JIPMER Campus Rd, Gorimedu
Dhanvantari Nagar
Puducherry-605 006.
(Dr. Rakesh Aggarwal)

Member (*ex-officio*) The President
Indian Drug Manufacturers Association
102-B, Poonam Chambers, 'A' Wing'
Dr. Annie Besant Road, Worli
Mumbai – 400 018.
(Shri Mahesh H. Doshi)

Member (*ex-officio*) The President
Organization of Pharmaceutical Producers of India (OPPI),
Peninsula Chambers, Ground floor
Ganpat Rao Kadam Marg, Lower parel
Mumbai- 400 013.
(Shri S. Sridhar)

Member (*ex-officio*) The President
Indian Pharmaceutical Alliance (IPA)
Mumbai.
(Shri Samir Mehta)

Member-Secretary (*ex-officio*) The Secretary-cum-Scientific Director
Indian Pharmacopoeia Commission
Sector-23, Raj Nagar
Ghaziabad-201 002.
(Dr. Rajeev Singh Raghuvanshi)

The Scientific Body

The composition of the Scientific Body is given below:

Chairperson Prof. N. K. Ganguly
Former Director General, ICMR
C-191, 9th Floor, City View Apartment
Plot No. 199, Sector-35
Noida-201 301.

Member Dr. Nitin Bhatia
Associate Vice President
(Tech. & Veterinary Regulatory)
Intas Animal Health
Intas Pharmaceuticals Ltd.
Corporate House
Near Sola Bridge, S. G. Road, Thaltej
Ahmedabad-380 054.

Member Prof. Naresh Bhatnagar
Department of Mechanical Engineering
Indian Institute of Technology,
Delhi, Hauz- Khas,
New Delhi -110 016.

Member Prof. Vinod Kumar Dixit
Former Dean, Faculty of Pharmacy &
Retired Professor of Pharmacognosy
Dr. Hari Singh Gour University,
Sagar - 470 003.

Member Dr. Sunil Gairola
Executive Director
Serum Institute of India Pvt. Ltd.,
212/2, Hadapsar,
Pune - 411 028.

Member Dr. Nithya Gogtay
Associate Professor
Department of Clinical Pharmacology
KEM and Seth G. S. Medical College
Mumbai - 400 012.

Member Prof. Ramesh. K. Goyal
Vice Chancellor
Delhi Pharmaceutical Sciences & Research
University
M.B.Road, Pushp Vihar, Sector 3
Delhi - 110 017.

Member Dr. Praveen Khullar
Head of MSAT, GenMed Cluster
Europe & International, IA GenMed
Sanofi Healthcare (India) Pvt. Ltd.
L-121, Phase III A, GIDC
Verna Industrial Estate
Verna, Goa - 403 722.

Member Dr. Arun Kumar Mishra
Head, Global Regulatory
Affairs-Health & Wellbeing
Unilever Ltd.,
Gurugram - 122 001.

Member Dr. Amulya K. Panda
Former Director
National Institute of Immunology
Aruna Asaf Ali Marg
New Delhi – 110 067.

Member Shri A. K. Pradhan
Joint Drugs Controller (I)
CDSCO (HQ)
FDA Bhawan, Kotla Road
New Delhi-110 002.

Member Dr. D. Srinivasa Reddy
Director
Indian Institute of Integrative Medicine
Post Bag No. 3, Canal Road
Jammu-180 001.

Member Dr. V. Satyanarayana
Chairman & Managing Director
M/s. Sipra Labs Ltd.
7-2-1813/5/A Industrial Estate
Sanath Nagar
Hyderabad- 500 018.

Member Dr. Hemant Kumar Sharma
President
Research & Development
APL Research Centre II
(A Division of Aurobindo Pharma Ltd.)
Survey No. 71&72, Indrakaran (V.)
Sangareddy (M)
Medak District -502 329.

Member Dr. A. K. Singh
Chief Executive Officer
Biotech Park
Sector G, Jankipuram, Kursi Road,
Lucknow - 226 021.

Member Dr. Raman Mohan Singh
Director
Central Drugs Testing Laboratory
Zonal FDA Bhawan, Bellasis Road
Govt. Medical Stores Depot Compound
Mumbai Central
Mumbai-400 008.

Member Prof. Sanjay Singh
Vice Chancellor
Babasaheb Bhimrao Ambedkar University
(A Central University)
Vidya Vihar, Raebareli Road
Lucknow-226 025.

Member	Dr. Rakesh Narayanrao Tirpude Assistant Commissioner Food & Drugs Administration Maharashtra State Mumbai- 400 051.
Member	Dr. Anil Kumar Tyagi CSO, R&D Department Mankind Pharma Ltd. 208, Okhla Industrial Estate, Phase III New Delhi – 110 020.
Member	Shri Salim A. Veljee Former Director Directorate of Food & Drugs Administration 'Dhanwantari', Opp. Shrine of the Holy Cross Bambolim Goa 403 202.
Member	Dr. Ram A. Vishwakarma Former Director Indian Institute of Integrative Medicine Post Bag No. 3, Canal Road Jammu-180 001.
Member	Prof. Naveet Wig Head, Department of Medicine All India Institute of Medical Sciences-Delhi Ansari Nagar New Delhi - 110 029.
Member-Secretary (ex-officio)	The Secretary-cum-Scientific Director Indian Pharmacopoeia Commission Sector-23, Raj Nagar Ghaziabad-201 002 (Dr. Rajeev Singh Raghuvanshi)

Expert Working Groups

Review of IP Work

Prof. C.K. Kokate, Dr. D.B. Anantha Narayana, Dr. Nitya Anand, Mr. J. L. Sipahimalani, Prof. Rama Shanker Verma, Mr. R. Sridharan, Mr. R. Raghunandanan, Prof. Saranjit Singh, Mr. Vinod Arora.

Sub-Group for Review of IP Work

Dr. Ajay Sachan, Mr. Gaurang Oza, Dr. N. Murugesan, Dr. Raman Mohan Singh, Mr. S. L. Jat, Mr. S.S. Venkatkrishnan.

Active Pharmaceutical Ingredients

Dr. Srinivasa Reddy, Dr. Raman Nanduri, Dr. Rajasekhar Akkaraju, Dr. Suryanarayana Mulukutla, Dr. Ch. V.V. Raju.

Allergen

Dr. S.N. Gaur, Prof. M.K. Agarwal, Dr. Surya Kant, Dr. A.K. Jain, Dr. Naveen Arora, Dr. W.A. Shaikh, Dr. Nagendra Prasad, Dr. S.E. Reddy, Dr. Jai Prakash, Dr. Achla Prasad.

Anti-Cancer

Mr. Ajay Shankar Singh, Mr. Bobby George, Mr. Mohan Jain, Dr. N. Padmaja, Dr. S. D. Sinha, Dr. Sanjeev Kumar.

Anti-Retroviral

Dr. Shripad D. Banavali, Dr. Imtiyaz Basade, Dr. Manish Gangrade, Dr. Nilesh Jha, Dr. Kishor Shenoy P., Dr. Radhay Shyam Gupta, Dr. K. Rathnakar Reddy.

Anti-Sera

Dr. Krishna Mohan B., Dr. Aldon Fernandes, Dr. Sumant Karnik, Ms Leena Menghaney, Dr. Amulya K. Panda, Dr. Nitin Salvi, Dr. Anil Yadav, Dr. Anand Zachariah, Nominee of CDL, Kasauli, Nominee of CRI, Kasauli, Nominee of CDSCO and Nominee of IPC.

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Dr. Surinder Singh, Dr. A. Ramkishan, Dr. Jaspreet Singh, Dr. C. Nirmala Raju, Ms. Meenu Batolar, Mr. Parveen Jain, Dr. Rahul Kulkarni, Prof. Rama Shanker Verma, Dr. Sriram Akundi, Dr. Samir Sangitrao, Dr. V. G. Somani, Dr. Rajesh Singh.

Blood and Blood Related Products

Dr. Debasish Gupta, Dr. J. P. Prasad, Ms. Kanchan Ahuja, Dr. Ranjeet S. Ajmani, Dr. Rajendra Chaudhary. Dr. Ratti Ram Sharma.

Clinical Medicine and Pharmacology

Prof. Y. K. Gupta, Dr. C. B. Tripathi, Prof. Praveen Agrawal, Prof. Rakesh Yadav, Dr. Pooja Gupta.

Excipients

Dr. Anil Kumar Tyagi, Dr. Ajaya Prakash, Dr. Ashok Panwar, Dr. P.B.N. Prasad, Dr. Rajasekhar Akkaraju, Dr. Subodh Priolkar.

Fixed Dose Combinations

Dr. Praveen Khullar, Dr. Rajiv Desai, Mr. Rahul Dev, Mr. Sanjeev Kumar, Dr. S. M. Mudda, Dr. Subhash C. Mandal, Ms. Sheetal Chadha, Dr. V. G. Somani.

General Chapters

Dr. Alka Mukne, Dr. Kundan Patil, Dr. Pramod Dalvi, Prof. Sanjay Singh, Dr. S. K. Balaji, Mr. S. L. Jat, Dr. Shailendra Saraf, Dr. Vinay G. Nayak.

General Chapter on Dosage Form

Dr. Amit Mishra, Mr. Anthony Gomes, Dr. Deo Narain Dikshit, Dr. Hemal Patel, Dr. N. Murugesan, Dr. Prashant Dikshit, Mr. Vinod Arora.

Herbal Products

Mr. A. K. Pradhan, Dr. S.K. Barik, Dr. D.B. Anantha Narayana, Prof. Kanchan Kohlli, Dr. Neeraj Tandon, Dr. Neelima Kshirsagar, Dr. Ram Vishwakarma, Prof. R. K. Goyal, Prof. S. S. Handa, Prof. Shridhar Dwivedi, Dr. Sanjeev Tripathi.

Impurity Standard Review

Dr. Anil Kumar Tyagi, Dr. D.P. Pathak, Dr. Hemant Kumar Sharma, Dr. P. R. Upadhyay, Prof. Saranjit Singh, Dr. S. K. Dubey, Dr. Vinod Kumar Kansal.

IPRS Review

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Dr. J. P. Mehta, Dr. Vaishali N. Patel, Mr. Parikh Anant G., Mr. C. Hariharan, Dr. P.K. Chitnis, Dr. Suhas Mangaonkar, Mr. Nilesh Shinde Prabhakar, Mr. Om Prakash Verma, Dr. R. Pirabhakaran, Mr. Ashok Desai.

Ophthalmic

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Dr. Arun Bhardwaj, Dr. K. Anand Kumar, Dr. Krishna Mohan, Dr. Mahesh K. Bhalgat, Dr. Parag Nagarkar, Dr. Rajesh Jain, Late Dr. Suresh Jadhav, Dr. V.G. Somani.

Veterinary Products

Prof. S.K. Garg, Dr. Praveen Malik, Mr. Rajesh Kumar Verma, Dr. R.K. Singh, Dr. B.P. Srinivasa, Dr. S.M. Byregowda, Dr. A.K. Tiwari, Dr. B. Mohana Subramanian, Dr. R.P. Singh, Dr. N.K. Mahajan, Dr. Arun Atrey, Dr. Sandeep Singh, Dr. V.A. Srinivasan, Dr. G.S. Reddy, Dr. B. Piruthiviraj, Dr. D.J. Kalita, Mr. Nitin Bhatia, Mr. Sanjay Gavkare, Dr. T.V.S. Rao.

Vitamins, Minerals, Amino Acids, Fatty Acids

Mr. Sanjeev Kumar, Dr. B. Dinesh Kumar, Dr. Parvinder Pal Singh, Mr. Sunil Bakshi, Mr. Gaurang Oza, Mr. Rajasekhar Akkaraju, Mr. Sudama Prasad, Mr. Girish Juneja, Mr. Deepak Arora and Dr. Hemal Patel.

IP Digitalization

Mr. Atanu Roy, Mr. Saurab Biswas, Ms. Bharati Khanna, Mr. Rajender Singh and Nominee from CDSCO.

Websites

Dr. Arun Kumar Mishra, Dr. D.B. Anantha Narayana, Dr. Gurpreet Sandhu, Prof. R. K. Goyal, Mr. R. Chandashekhar.

IPC Secretariat and Staff

Scientific and administrative support to the Expert Working Groups has been extended by the following staff of the IPC.

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Dr. Rajeev Singh Raghuvanshi

Personnel Staff to the Secretary-cum-Scientific Director

Mr. Munendra Kumar Poonia, Mr. Bhupendra Kumar and Mr. Surinder Kumar.

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Reference Standard Division

Dr. Robin Kumar, Dr. Anuj Prakash, Dr. Manisha Trivedi, Mr. Arvind Kumar Sharma, Ms. Priyanka Nagar, Ms. Ashu Rathi, Mr. Manoj Kumar Bagri, Mr. Suryadev Sharma, Dr. Prachi Rathi, Dr. Divya Kaushik, Ms. Pooja Nagpal, Dr. Anupama Mittal, Ms. Natasha Pathak, Mr. Abhishek, Ms. Nupur, Mr. Mahesh Chand, Ms. Smriti Rawat, Ms. Jayati Sikarvar, Ms. Hema Kapoor, Mr. Churchill Rana, Mr. Bharat Veer, Mr. Gaurav Kumar, Ms. Shiksha Kaushik, Ms. Menka Pathak, Ms. Neeti Pandey, Mr. Rahul Kumar Kadiyan, Ms. Parul Choudhary, Mr. Ankush Dhangi, Ms. Chhaya Tiwari, Ms. Vidushi Kansal, Ms. Sheenu Gautam, Ms. Anshika

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Biologics Division

Dr. M. Kalaivani, Ms. Anubhuti Goyal, Dr. Priyanka Chaudhary, Dr. Prachi Pathak, Ms. Swati Tyagi and Mr. Arjun.

Microbiology Division

Dr. Anil Kumar Teotia, Dr. Manoj Kumar Pandey, Dr. Prasad Thota, Mr. Piyush Kumar, Ms. Roshni Rajpali, Ms. Bhavna Kumari, Ms. Ekta Badhgothri, Mr. Sachin Kumar, Mr. Mukesh Kumar and Mr. Sunder.

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Other Participants

Participants other than those mentioned above who assisted in the work relating to preparation of this edition are given below:

A. Meenakshi, M/s Madras Pharma
Dr. A.G. Seshadrinathan, M/s Shreya Life Sciences Pvt. Ltd.
A.K. Bhatnagar, Bureau of Indian Standards
Adhir Chaubal, M/s Indian Immunologicals Ltd.
Aditi Kambli, Bharat Serums & Vaccines Ltd.
Ajay Kumar Kundu, M/s GSK Pharmaceuticals Ltd.
Ajay Tyagi, M/s Swiss Garnier Life Sciences
Ajit Singh, IDMA
Akhilesh Jain, M/s Shreya Life Sciences Pvt. Ltd.
Altaf Shaikh, M/s Medispray Laboratory Pvt. Ltd.
Amarpreet Kaur, M/s Sun Pharmaceutical Industries Ltd.
Dr. Amit Agarwal, M/s Natural Remedies Pvt. Ltd.
Amit Khandhar, M/s Zydus Healthcare Ltd.
Amit Patel, M/s Vaibhav Analytical Services
Anil Kumar Tripathi, M/s Morepen Laboratories Ltd.
Anil P. Vichare, M/s Paras Organics
Aniruddha Chalke, M/s Cipla Ltd.
Anjali Tawde, M/s Cipla Ltd.
Anoop Tandon, Gas Industries Association
Anurag Mishra, M/s IPCA Laboratories Ltd.
Apoorva Anand, NIB, Noida
Aradhana Saini, M/s ITL Labs Pvt. Ltd.

Archana Singh, M/s Marc Laboratories Ltd.
Arindam Das, M/s Linde India
Arjun Singh Gohil, M/s Alembic Pharmaceuticals Ltd.
Arun Kulkarni, M/s Emcure Pharmaceuticals Ltd.
Arun Kumar Singh, M/s Lupin Ltd.
Dr. Arvind M. Badiger, M/s BDR Pharmaceuticals (I) Pvt. Ltd.
Dr. Arvind Bansal, NIPER, Mohali
Asheesh Kumar Bandopadhyay, M/s Strassenburg Pharmaceuticals Ltd.
Asheesh Sharma, M/s Aristo Pharmaceuticals Pvt. Ltd.
Ashok Kumar Sharma, M/s Symbiotec Pharmalab Pvt. Ltd.
Ashok Rokde, M/s Khandelwal Laboratories Pvt. Ltd.
B. Jayashree, M/s Madras Pharmaceuticals
Dr. B. L. Agarwal, M/s Highkon Pharma
Dr. B. M. Nagaraju, M/s Allergen India Ltd.
B. N. Sharma, M/s Ravenbhel Healthcare Pvt. Ltd.
B. Venkata Subbaiah, M/s Dr. Reddy's Laboratories Ltd.
B.M. Jaya Rao, M/s Hetero Drugs
Balkrishna Sawant, M/s Medispray Pvt. Ltd.
Basant Rana, M/s Tirupati Medicare Ltd.
Bikash Roy, CDSCO
Bhavesh Shah, M/s Alembic Pharmaceuticals Ltd.
Dr. Binod Kumar Das, M/s Amoli Organics Pvt. Ltd.,
Brahma Rao, M/s Celon Labs.
Dr. C.K. Katiyar, M/s Emami Ltd.
Catherine Raphael, M/s IPCA Laboratories Ltd.
Dr. Chaitanya Kumar Koduri, USP- India (P) Ltd.
Chandra Shekhar Pawar, CDSCO
Charanjeet Chitkara, M/s Morepen Laboratories Ltd.
Dr. Charu M. Kamal, NIB, Noida
Dr. Chetan Bhavsar, M/s BDR Pharmaceuticals (I) Pvt. Ltd.
Chetan Gupta, M/s Emcure Pharmaceuticals Ltd.
Dr. Chintan Dholakia, M/s Zydus Cadila
D. S. Jadhav, M/s Macleods Pharmaceuticals Ltd.
Dr. D.P. Ghosh, M/s Albert David Ltd.
Dadasaheb V Pangavhane, M/s Cipla Ltd.
Dalia Bandyopadhyay, M/s Ajanta Pharma Ltd.
Deepak Kasbi, M/s Lupin Ltd.
Deepak Kumar Arora, M/s Sun Pharmaceutical Industries Ltd.
Deepak Nagar, M/s Synokem
Deepak Vig, M/s Acme Generics Ltd.
Deepti Nair, M/s GSK Pharmaceuticals
Devesh Kumar Tiwari, M/s USV Pvt. Ltd., Baddi
Dhananjay Gagare, M/s Wockhardt Research Centre
Dharmin Joshi, M/s Intas Pharmaceuticals Ltd.
Dhiraj Chugh, M/s Zenax Animal Health Pvt. Ltd.
Dhruvil Patel, M/s Sun Pharmaceutical Industries Ltd.
Dr. Dilip Dhore, M/s Natco Pharma Ltd.
Dilip Kumar Kulkarni, M/s Lupin Ltd.
Dilip Kumar Midya, M/s Dr. Reddy's Laboratories Ltd.
Dilip Patil, M/s Sun Pharmaceutical Industries Ltd.
Dinesh Chirputkar, M/s Mylan Laboratories
Dinesh Kumar Varshney, M/s Supermax Drugs & Pharmaceuticals (P) Ltd.
Dinesh Reddy. K, M/s Hetero Labs Ltd.
Dipak Dhote, M/s Blue Cross Laboratories Pvt. Ltd.
Dipak Saptarshi, M/s Intas Pharmaceuticals Ltd.
Dr. Dileep Bhosale, M/s Glenmark Pharma
Divya Kumar Bora, M/s Lupin Ltd.
Dhananjay Brahmbhatt, M/s Harson Laboratories
Durga Prasad Reddy, M/s Gland Pharma Ltd.
Faiza Lambe, M/s Cipla Ltd.
Faslu Rahman P, M/s Terumo Penpol Pvt. Ltd.
G. Satya Srinivasu, M/s Troikaa Pharmaceuticals Ltd.
G.S. Tiwari, M/s Windlas Healthcare Pvt. Ltd.
G.V.S. Rao, M/s Micro Labs Ltd.
Gajanan B. Kasawar, M/s Dr. Reddy's Laboratories Ltd.
Ganadhish Kamat, M/s Lupin Ltd.
Dr. Gauri Misra, NIB, Noida
Gautam Panchwadker, M/s Cipla Ltd.
Dr. George Patani, M/s Inga Laboratories P. Ltd.
Girish Deshpande, M/s Mylan Lab. Ltd.
Gopal Singh, M/s Bharat Biotech
Dr. Gopal Vaidyanathan, M/s Natco Research Centre
Dr. Gopalkrishna Lakki Reddy, M/s Dr. Reddy's Lab. Ltd.
Gunwant Singh Bind, M/s East African India Overseas
Gunwant Singh Desai, M/s Zydus Cadila
Gurminder Bindra, NIB, Noida
Guruprasad Naik, M/s Sanofi India Ltd.
Gyanendra Kumar, M/s Mankind Pharma Ltd.
H.P. Jhala, M/s J.B. Chemicals & Pharmaceuticals Ltd.
Dr. Hantz Tattegrain, M/s Pfizer Inc.
Hardik Shah, M/s Accurate Pharmaceuticals
Hareesh Kumar V., M/s Biocon Ltd.
Dr. Harish Chander, NIB, Noida
Harish Jain, Federation of Pharma Entrepreneurs-India
Harit Kasana, NIB, Noida
Harshad Satpute, M/s Vaibhav Analytical Services
Harshit Mehta, M/s Medico Remedies
Harsit Kumar, M/s Rhydburg Pharmaceuticals Ltd.
Himanshu Khatri, M/s Troikaa Pharmaceuticals Ltd.
Dr. Imtiyaz Basade, M/s Mylan Laboraories Ltd.
Indhushri, M/s Glaxo SmithKline Pharmaceuticals Ltd.
Dr. Indu Bhushan, M/s Steerlife India Pvt. Ltd.
Intkhab Alam, M/s Unicure (India) Pvt. Ltd.
J. Gopalakrishnan, M/s Sun Pharmaceutical Industries Ltd.

Jagabandhu Panda, M/s Megafine Pharma (P) Ltd.
Jagdish C. Koshti, M/s Blue Cross Laboratories Ltd.
Jagdish Kumar, Federation of Pharma Entrepreneurs-India
Jaipal Meena, NIB, Noida
Jatin Thakkar, M/s Gelnova Laboratories Pvt. Ltd.
Jayesh Kumar Patel, M/s Sovereign Pharma Pvt. Ltd.
Jayesh Pandya, M/s Suyog Life Sciences Pvt. Ltd.
Jeena Cyril, M/s Sanofi India Ltd.
Jejesh Birje, M/s Johnson & Johnson Pvt. Ltd.
K. Ravinder Reddy, M/s Maithri Laboratories Pvt. Ltd.
K.V. Subbarao, M/s Natco Pharma Ltd.
Kamaraj Abraham, M/s Recipharm Pharmaservices Pvt. Ltd.,
Kamlesh Bhatt, M/s Sun Pharmaceutical Industries Ltd.
Dr. Kamlesh Dutta, M/s Quest Pharmaceuticals (P) Ltd.
Kamles S. Rajput, M/s Amoli Organics Pvt. Ltd.
Kanduri Srinivas, M/s Dr. Reddy's Laboratories Ltd.
Dr. Karuna Shanker, CSIR-CIMAP, Lucknow
Kavita Gajinkar, M/s Cipla Ltd.
Ken Furlong, M/s NutraSweet Co.
Keshav Kumar, M/s Reckitt Healthcare
Kiran Ainapure, M/s Glaxo SmithKline Pharmaceuticals Ltd.
Kiran Rajbhar, M/s Novartis Healthcare Pvt. Ltd.
Kiranyamayee Chitnis, M/s Glenmark Pharmaceuticals Ltd.
Krishnakant Vichare, M/s Medley Pharmaceuticals Ltd.
Krupa Parikh, M/s Torrent Pharmaceuticals Ltd.
KSV Prasada Raju, M/s Vasudha Pharma Chem Ltd.
Kuldeep Gupta, M/s Vivek Pharmachem (India) Ltd.
Kunal Shah, M/s Sun Pharmaceutical Industries Ltd.
Dr. Laila S. Rajwani, M/s Sovereign Pharma
Lalit Kumar Tyagi, M/s Mankind Research Centre
Lal Singh Negi, M/s Akums Pharma
M. Madan Mohan Reddy, M/s Eisai Pharm. India Pvt. Ltd.
M. Madhusudhan, M/s Divis Laboratories Ltd.
Madhvan P., M/s Dr. Reddy's Laboratories Ltd.
M. Venkata Narayana, M/s MSN Life Sciences Pvt. Ltd.
M.K. Raja Sekar, M/s Orchid Chemicals & Pharmaceutical Ltd.
M.M. Agashiwala, M/s Kilitch Healthcare India Ltd.
M.M. Sarvankar, M/s Sanjivani Parenteral Ltd.
M.T. Dave, M/s Gufic Biosciences Ltd.
Mahaveer Mugad, M/s HLL Lifecare Ltd.
Mahendra Bisht, M/s Innova Captab Pvt. Ltd.
Mahendra K. Sharma, M/s Cachet Pharmaceuticals Pvt. Ltd.
Mahendra Singh, M/s Zydus Cadila Ltd.
Mahesh V. Patil, M/s Mac-Chem Products (India) Pvt. Ltd.
Dr. Malati Vakamulla, M/s Dr. Reddy's Laboratories Ltd.
Manali Apshinge, M/s IPCA Laboratories Ltd.
Dr. Maneesh Singhal, AIIMS
Dr. Manish Kumar, M/s Biological E Ltd.
Manjunath C. Patil, M/s Biocon Ltd.
Manjunath M. S, M/s Micro Labs Ltd.
Manoj Kamra, M/s Cadila Healthcare Ltd.
Manoj Kumar, M/s Ear India
Dr. Manoj Kumar Singh, M/s Micro Labs Ltd.
Marut Shandilya, M/s Tirupati Medicare Ltd.
Maruti Sapatale, M/s Mac-Chem Products (India) Pvt. Ltd.
Mayur Gurjar, M/s Cadila Healthcare Ltd.
Dr. Meena Kumari, NIB, Noida
Meena Shah, Independent Consultant
Meghana Jagtap, M/s Bajaj Healthcare Ltd.
Mihir Shah, M/s IPCA Laboratories Ltd.
Dr. Milind Joshi, M/s J.B. Chemicals & Pharmaceuticals Ltd.
Minhal Husain, M/s ZIM Laboratories Ltd.
Dr. Mohd. Idris, CSIR-CCMB, Hyderabad
Mohd. Manzar Alam, M/s Ravenbhel Healthcare Pvt. Ltd.
M. S. Rahman, M/s Panacea Biotech Ltd.
Mukesh Gupta, M/s IPCA Laboratories Ltd.
Mukesh K. Sharma, M/s Kimia Biosciences Ltd.
N. Kamalakar Rao, M/s Swiss Garnier
N. Nanda Gopal, NIB, Noida
N.N. Raut, M/s Neon Labs Ltd.
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Nandlal Yadav, M/s USV Ltd.
Naresh Gupta, M/s Aarti Drugs
Naresh Tondare, M/s Dr. Reddy's Laboratories Ltd.
Naveen Yadav, CDSCO, New Delhi
Navneet Mehta, M/s Laurus Labs Ltd.
Navneet Tewatia, Indian Pharmaceutical Association
Nayyar Zulfiqar, M/s Astra Zeneca Pharma India Ltd.
Neelam Tarani, M/s Sun Pharmaceutical Industries Ltd.
Neeraj Taggar, M/s Ind-swift Ltd.
Neeta Joshi, M/s Neon Laboratories Ltd.
Nguyen Thi Van Thao, M/s Abbott Ltd.
Dr. Nidhi Sandal, INMAS
Nilesh C. Mehendale, M/s Janssen Pharma
Dr. Nikhil Singh, M/s Zydus Cadila
Nilesh Saluja, M/s Mylan Laboratories Ltd.
Nimit Panchal, M/s BDR Pharmaceuticals (I) Pvt. Ltd.
Nishant Parsania, M/s Sun Pharmaceutical Industries Ltd.
NNVVSS Narayana Murthy, M/s Dr. Reddy's Lab. Ltd.
Dr. Nripendra Mishra, NIB, Noida
Om Prakash Sule, M/s Windlas Biotech Ltd.
Omkar Nath Singh, M/s Sterile India
P. Durga Bhavani, M/s Dr. Reddy's Laboratories Ltd.
P.B. Kawade, M/s Glenmark Pharmaceuticals Ltd.
Pandurang Saratkar, M/s Pharmaforce Labs.
Pankaj Patel, M/s Vaibhav Analytical Services

Dr. Pankaj Sharma, NIB, Noida
Dr. Pankaj Sahu, M/s Sun Pharmaceutical Industries Ltd.
Parag Gadkari, M/s Inventia Healthcare Pvt. Ltd.
Paras Jain, NIB, Noida
Paresh Parmar, FDL, Gujarat
Dr. Parvinder P. Singh, CSIR-IIIM, Jammu
Payal Bhargava, M/s Mylan Laboratories Ltd.
Pritesh Dasare, M/s Sun Pharmaceutical Industries Ltd.
Pooja Bhalerao, M/s Labindia Instruments Pvt. Ltd.
Pradip V. Tarade, M/s Megafine Pharma (P) Ltd.
Prahladsinh J Rana, M/s Zydus Cadila Healthcare Ltd.
Pramod Naik, M/s Centaur Pharmaceuticals Pvt. Ltd.
Pranav Jogani, M/s Zydus Cadila
Prashant Gaonkar, M/s Procter & Gamble Health Ltd.
Prashant Sonawane, M/s SMAART Pharmaceuticals
Prathap. E, M/s Swiss Garnier Life Sciences
Praveen Jain, M/s Innova Captab Ltd.
Pravin Makwana, M/s Unique Pharmaceuticals Laboratories
Pravin Patil, M/s GSK Pharmaceuticals Ltd.
Pravina Kolwalkar, M/s Cipla Ltd.
Prem Singh Tomar, M/s Mylan Laboratories Ltd.
Pritesh Shah, M/s Troikaa Pharmaceuticals Ltd.
Pritesh Upadhyay, M/s Lupin Ltd.
Priya Mishra, M/s VXL Life Sciences
Priya Singh, M/s FDC Ltd.
Priyank Joshi, M/s Mankind Pharma Ltd.
Puja Agnihotri, M/s Sun Pharmaceuticals Industries Ltd.
Purnima N. Shah, M/s ACG Associated Capsules Pvt. Ltd.
Purshotam Lal, M/s Lupin Ltd.
Pushpendu Gaurav, M/s Karnataka Antibiotics and Pharmaceuticals Ltd.
Dr. R. K. Sanghavi, IDMA
R. Pradeep, M/s Maithri Drugs Pvt. Ltd.
R. Sridhar, M/s Apex Laboratories Pvt. Ltd.
R.M. Shah, M/s JB Chemicals & Pharmaceuticals Ltd.
R.P. Mishra, M/s PIL Pharmaceuticals Ltd.
Radhika Joshi, M/s ITL Labs Pvt. Ltd.
Rahul Chaudhary, M/s Psychotropics India Ltd.
Rahul Shakya, M/s Relax Pharmaceutical
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Dr. Rakesh Garg, AIIMS
Rakesh Kumar, M/s Ind-Swift Laboratories
Dr. Rakesh Mishra, CSIR-CCMB, Hyderabad
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Rubina Bose, CDSCO-WZ, Mumbai
Dr. Rupesh Kelaskar, M/s FDC Ltd.
Ryan Bittner, M/s Mylan Laboratories Ltd.
Dr. Sai R. Kasi Viswanathan, M/s Micro Labs Ltd.
Dr. S. Kumar, M/s Akums Drugs & Pharmaceuticals Ltd.
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Vijay Chandankar, CDSCO, New Delhi
Vijay Kumar Solanki, M/s Alembic Pharmaceuticals Ltd.
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Introduction

Introduction

The ninth edition of the Indian Pharmacopoeia (IP 2022) is published by the Indian Pharmacopoeia Commission (IPC) in accordance with a designed plan to fulfil the requirements of the Drugs and Cosmetics Act 1940 and Rules thereunder and completed through untiring efforts of the members Scientific Body of the IPC, members of the Expert working Groups, IP Secretariat and IPC Laboratory over a period of about one year. It supersedes the IP 2018 edition but any monograph of the immediately preceding edition that does not figure in this edition continues to be official as stipulated in the Second Schedule of the Drugs and Cosmetics Act, 1940.

Presentation

The Indian Pharmacopoeia 2022 is presented in four volumes. Volume I contain the Notices, Preface, about Indian Pharmacopoeia Commission, Acknowledgements, Introduction, General Notices, General Chapters, Reference Data and Index. Volume II contains the General Notices, General Monographs on Active Pharmaceutical Ingredients and Dosage Forms, Monographs on Drug substances, Dosage forms and Pharmaceutical aids (A to M). Volume III contains the General Notices, Monographs on Drug substances, Dosage forms and Pharmaceutical aids (N to Z) followed by and distinguished by colour codes, Monographs on Vitamins, Minerals, Amino Acids, Fatty Acids etc., Phytopharmaceuticals, Herbs and Herbal Products, Vaccines and Immunosera for Human Use, Blood and Blood Related Products, Biotechnology Derived Therapeutic Products, Allergen Products and Radiopharmaceutical Preparations. Volume IV contains the General Notices, Notices, Veterinary Monographs and Index.

Format

In an effort to make the pharmacopoeia more user-friendly, the design of the texts of the monographs and test methods are kept same. Cross-referencing between the individual monographs has been avoided to make each monograph complete in itself, thus making it convenient to the user.

Pharmacopoeial Requirements

As in the past, this compendium provides a publicly available statement concerning the quality of a product that can be expected and demonstrated at any time throughout the accepted shelf-life of the article. The standards laid down represent the minimum with which the article must comply and it is for the manufacturer to ensure that the article is manufactured in accordance with Good Manufacturing Practices (GMPs). It is essential that sufficiently stringent limits are applied at the time of release of a batch of drug substance or drug product so that the pharmacopoeial standards are met until its expiry when stored under the storage conditions specified.

It must be noted that a valid interpretation of any requirement of the Indian Pharmacopoeia should be done in the context of the monograph as a whole, the relevant general monographs, where appropriate, the specified tests and methods of analysis including any reference to the relevant General Notices. Familiarity with the General Notices will facilitate the correct application of the requirements.

Changes

Keeping in view the essential requirement under the Drugs and Cosmetics Act, 1940 and Rules thereunder, the information on the category of a drug, and available usual strengths of dosage forms has been kept in this edition.

A total of 92 new monographs have been added in the current edition. Among the included monographs, 60 chemical, 21 Vitamins, Minerals, Amino acids, Fatty Acids etc., 03 Biotechnology Derived Therapeutic products, 04 Vaccines and Immunosera for human use, 02 Blood and Blood related products, 02 Herbs and Herbal products have been added in this edition.

The current edition also includes additional antiretroviral drug substances and formulations, anticancer drug substances and formulations inclusive of commonly used fixed-dose combinations and drugs used for COVID-19 therapy. Standards for new drugs and drugs used under National Health Programmes and included in National List of Essential Medicines are added. The drugs as well as their formulations currently not used are omitted from this edition. The number of monographs of Drug substances, Dosage forms, Excipients, Vitamins, Minerals, Amino acids, Fatty Acids etc., Phytopharmaceuticals, Herbs and Herbal Products, Vaccines and Immunosera for Human, Blood and Blood related products and Biotechnology Derived Therapeutic products have been increased in this edition.

General chemical tests for identification of an article have been almost eliminated and more specific infrared and HPLC tests have been given emphasis. The concept of relying on published infrared spectra as a basis for identification has been continued.

The use of chromatographic methods has been greatly extended to cope up with the need for the specificity in assays and in particular, in assessing the nature and extent of impurities in drug substances and drug products. few

monograph existing dissolution, assays and related substances tests have been upgraded to liquid chromatography method in view to have the specificity and to harmonise with other Pharmacopoeias globally to the extent necessary. The up-gradation in this edition covers all the amendments released after publication of Addendum 2021 to IP-2018.

In order to focus on contemporary challenge a separate category of Phytopharmaceuticals have been created and 07 monographs of herbs and herbal products have been upgraded and shifted to Phytopharmaceuticals section. Some existing essential oils monographs, crude herbal drugs and extracts monographs have been revised under the section Herbs and Herbal Products in volume III.

For the first time the General requirements of monoclonal antibodies & monographs of monoclonal antibodies i.e. Rituximab drug substance and Rituximab Injection monographs have been incorporated in this edition. New Monographs of Vaccines and Immunosera for Human Use, Blood and Blood related products and Biotechnology Derived Therapeutic Products are added, and some were upgraded in view of the latest development of the technology in the field.

Abnormal toxicity test have been deleted from specific monographs and retained only in General requirements of Vaccines and Immunosera for Human Use. Test of Pyrogen have been replaced with Bacterial Endotoxin Test in monographs of Vaccines and Immunosera for Human Use.

A separate volume for veterinary monographs as Volume IV of this edition of Indian Pharmacopoeia has been designed to provide comprehensive information to those concerned with the quality control of veterinary medicines. Several monographs on chemicals along with a number of monographs on veterinary vaccines, diagnostics, immunosera and surgical materials have been given place in this volume. Many monographs just on the basis of usual strengths have been mentioned in this volume while complete monographs are mentioned in other volumes of IP.

The monograph on Lorcaserin Hydrochloride Hemihydrate and Lorcaserin Hydrochloride Tablets and general chapter Assay of Human Anti-D Immunoglobulin Method B and C have been omitted.

General Chapters

Volume I is devoted mainly to test methods that are applicable to all the articles of the pharmacopoeia and general information pertaining to the quality requirements of medicinal substances. It also includes reference data such as reference spectra, typical chromatograms etc. The test methods reflect the sophistication of analytical methodology and instrumentation. Analytical methods in general are in harmony with those adopted internationally for monitoring the quality of drugs. The steps taken for harmonization have been initiated by the need to cope with the increasing demand for drugs manufactured in the country to meet globally accepted standards.

The scope of the Indian Pharmacopoeia has been extended to include 12 new general chapters such as Microbiological Examination of *Burkholderia cepacia* complex in Non-sterile Products, Approach to Alternative Microbiological Methods, Design and Development of Biological Assay and its Validation, Subvisible Particulate Matter in Therapeutic Protein Injection, Protocol for determination of the PRP content of Haemophilus Type b Conjugate Vaccine by HPAEC-PAD, Adjuvants for vaccines, Test for absence of Mycobacteria.

The new chapter on Elemental Impurities and Nitrosamine Impurities have been included in this edition.

The general chapters on chromatographic techniques such as Ultraviolet and Visible Absorption Spectrophotometry, Gas Chromatography, Liquid Chromatography, Paper Chromatography, Thin-layer Chromatography and Inductively Coupled Plasma-Mass Spectrometry have been revised as per the technological requirements. The general chapter on Dissolution, friability of uncoated tablets, content of packaged dosage units, optical rotation and specific optical rotation have been revised as accepted internationally. The general chapter on General Identification reactions of ions and functional groups, Assay of oxygen, oxygen-flask method have been revised in accordance with advancement in the field. The General Monographs such as Active Pharmaceuticals Ingredients, Inhalation preparations, Parenteral preparations have also been revised.

The new General Monograph on Active Pharmaceutical Ingredients has been included in this edition.

The general chapters on Microbial contamination in Non-sterile Products, Microbiological assay of Antibiotics, Composition of polysaccharide vaccines, Cell substrates for the production of vaccines for human use, Extraneous agents in Viral Vaccines, Assay of Insulin and Assay of Human Anti-D Immonugloblin have been revised.

The trend towards controlling the microbial quality of all medicinal products has been recognized and the requirement regarding limits of microbial contamination, even of products for oral administration and topical application, so that adequate controls are exercised by the manufacturers by the adoption of GMPs has been continued.

Monographs

The General Monographs on Active Pharmaceutical Ingredients (APIs) and Dosage Forms are grouped together at the beginning of Volume II. They are followed by the

monographs for the APIs, pharmaceutical aids and individual dosage forms, all in alphabetical order up to Volume III. Monographs for other articles of special nature such as Vitamins, Minerals, Amino Acids, Fatty Acids etc., Phytopharmaceuticals, Herbs and Herbal Products, Vaccines and Immunosera for Human Use, Blood and Blood Related Products, Biotechnology Derived Therapeutic Products, Allergen Products and Radiopharmaceutical Preparations are given in separate sections in Volume III. Special emphasis has been given to Veterinary monographs adding new ones and updating the existing monographs in volume IV.

A list of 92 new monographs included in the 2022 edition of the Indian Pharmacopoeia is given below:

Admissions

General Chapters

2.2.29 Microbiological Examination of *Burkholderia cepacia* Complex in Non-Sterile Products

2.2.30 Approach to Alternative Micro -biological Methods

2.2.31 Design and Development of Biological Assay and its Validation

2.2.32 Subvisible Particulate Matter in Therapeutic Protein Injections

2.3.56 Assay of Calcium Pantothenate

2.4.47 Raman Spectrometry

2.5.4. (i) Uniformity of Dosage Units

2.7.14 Test for absence of Mycobacteria

2.7.15 Protocol for determination of the PRP content of Haemophilus Type b Conjugate Vaccine by HPAECPAD

2.7.16 Adjuvants for vaccines

5.10 Elemental Impurities

5.11 Nitrosamine Impurities

General Monographs

Active Pharmaceutical Ingredients

General Requirements

Therapeutic Monoclonal Antibodies for Human Use

Monographs on Drug substances, Dosage forms and Pharmaceutical aids

2-Deoxy-D-Glucose

2-Deoxy-D-Glucose Sachet

Amifostine

Amifostine for Injection

Amlodipine and Valsartan Tablets

Apremilast

Apremilast Tablets

Aprotinin Injection

Azithromycin Eye Drops

Bosutinib

Bosutinib Tablets

Brivaracetam

Brivaracetam Tablets

Ceftriaxone and Sulbactam for Injection

Desogestrel

Desogestrel and Ethinyl Estradiol Tablets

Dextran 1

Dextran 40

Dextran 70

Dextropropoxyphene Hydrochloride and Paracetamol Tablets

Diclofenac Potassium

Diclofenac Potassium Tablets

Epalrestat

Epalrestat Tablets

Estradiol Hemihydrate

Ethyl Acetate

Ethynodiol Diacetate

Ethynodiol Diacetate and Ethinyl Estradiol Tablets

Fexofenadine Hydrochloride and Pseudoephedrine Hydrochloride Prolonged-release Tablets

Glipizide and Metformin Tablets

Itraconazole

Lenvatinib Mesylate

Lenvatinib Capsules

Mesna Tablets

Oxetacaine

Polymyxin B Sulphate

Polymyxin B for Injection

Prasugrel and Aspirin Gastro-resistant Capsules

Repaglinide and Voglibose Tablets

Ribavirin Capsules

Risperidone Syrup

Rocuronium Bromide

Rocuronium Injection

Sodium Starch Glycolate (Type B)

Sofosbuvir

Sofosbuvir and Daclatasvir Tablets

Sofosbuvir Tablets

Sugar Spheres

Tofacitinib Citrate

Tofacitinib Tablets

Trazodone Hydrochloride

Trazodone Tablets

Teneligliptin and Metformin Hydrochloride Prolonged-release Tablets

Triamterene and Hydrochlorothiazide Tablets

Valacyclovir Hydrochloride

Valacyclovir Tablets

Valganciclovir Hydrochloride

Valganciclovir Tablets

Vildagliptin and Metformin Tablets

Zanamivir

Vitamins, Minerals, Amino Acids, Fatty Acids etc.

Oil-Soluble Vitamins Capsules

Oil-Soluble Vitamins Oral Solution

Oil-Soluble Vitamins Tablets

Water-Soluble Vitamins Capsules

Water-Soluble Vitamins Tablets

Alpha Lipoic Acid

Biotin
Calcium Citrate Malate
Chromium Picolinate
Copper Gluconate
Glutamic Acid
Inositol
Lutein
Lysine Hydrochloride
Phenylalanine
Selenomethionine
Selenious Acid
Threonine
Tryptophan
Valine
Zinc Citrate

Phytopharmaceuticals

General Requirements on Phytopharmaceuticals

Herbs and Herbal Product Monographs

Chitrak
Siris

Vaccines and Immunosera for Human Use

Diphtheria, Tetanus, Acellular Pertussis, Hepatitis B, Inactivated Poliomyelitis and Haemophilus Influenzae Type b Conjugate Vaccine Adsorbed
Meningococcal Group A, C, W135 and Y Conjugate Vaccine
Diphtheria, Tetanus, Pertussis (Whole cell), Hepatitis B, Inactivated Poliomyelitis and Haemophilus Influenzae Type b Conjugate Vaccine Adsorbed
Bivalent Poliomyelitis Vaccine Type 1 & 3, Live (Oral)

Blood & Blood Related Products

Anti-D Blend (IgM + IgG) Monoclonal Reagents
Anti-D (IgG) Monoclonal Reagents

Biotechnology Derived Therapeutic Products

Rituximab
Rituximab Injection
Teriparatide Concentrated Solution

Upgradation

General Notices

Rounding rules for Calculation of Results

General Chapters

2.2.9 Microbial Contamination in Non-sterile Products
2.2.10 Microbiological Assay of Antibiotics
2.3.1 General Identification Reactions of Ions and Functional Groups
2.3.33 Assay of Oxygen
2.3.34 Oxygen-Flask Method
2.3.46 Assay of Insulins
2.4.7 Ultraviolet and Visible Absorption Spectrophotometry
2.4.13 Gas Chromatography
2.4.14 Liquid Chromatography
2.4.15 Paper Chromatography
2.4.17 Thin-layer Chromatography
2.4.22 Optical Rotation and Specific Optical Rotation
2.4.26 Solubility
2.4.42 Inductively Coupled Plasma Spectrometry
2.5.2 Dissolution
2.5.5 Friability of Uncoated Tablets
2.5.6 Content of Packaged Dosage Units
2.7.1 Composition of Polysaccharide Vaccines
2.7.2 Cell Substrates for the Production of Vaccines for Human Use
2.7.3 Extraneous Agents in Viral Vaccines
2.8.2 Assay of Human Anti-D Immunoglobulin
3.1 Infra-red Reference Spectra
3.2 Thin Layer Chromatograms of Phytopharmaceuticals, Herbs and Herbal Products
3.2.1 Thin Layer Chromatograms of Phytopharmaceuticals
3.2.2 Thin Layer Chromatograms of Herbs and Herbal Products
3.3 Liquid/HPTLC/Gas Chromatograms of Phytopharmaceuticals, Herbs and Herbal Products
3.3.1 Liquid/HPTLC/Gas Chromatograms of Phytopharmaceuticals
3.3.2 Liquid/HPTLC/Gas Chromatograms of Herbs and Herbal Products
4.1 Buffer Solutions
4.2 General Reagents
5.5 Impurities
5.9 Reference Substances

General Monographs

Capsules
Inhalation Preparations
Parenteral Preparations

Monographs on Drug substances, Dosage forms and Pharmaceutical aids

Amantadine Capsules
Amiloride and Hydrochlorothiazide Tablets
Aminocaproic Acid Tablets
Amitriptyline Tablets
Amlodipine Tablets
Ammonium Chloride
Amoxycillin and Potassium Clavulanate Oral Suspension
Amphotericin B
Amphotericin B Injection
Anastrozole Tablets
Anticoagulant Citrate Dextrose Solution
Anticoagulant Citrate Phosphate Dextrose Solution
Artemether and Lumefantrine Tablets
Aspartame

Aspirin Gastro-resistant and Atorvastatin Capsules
Aspirin Gastro-resistant and Rosuvastatin Capsules
Aspirin Tablets
Soluble Aspirin Tablets
Atenolol and Chlorthalidone Tablets
Atenolol Tablets
Atomoxetine Hydrochloride
Atosiban Acetate
Atracurium Besylate Injection
Azelnidipine
Azelnidipine Tablets
Bacitracin
Bacitracin Zinc
Benzoic Acid
Betamethasone Injection
Betamethasone Sodium Phosphate Tablets
Bicalutamide
Bicalutamide Tablets
Bisacodyl
Bromhexine Hydrochloride
Bromhexine Tablets
Bromocriptine Capsules
Bromocriptine Tablets
Bumetanide Tablets
Buprenorphine and Naloxone Sublingual Tablets
Buprenorphine Hydrochloride
Buprenorphine Sublingual Tablets
Calamine
Capreomycin Sulphate
Capreomycin Injection
Captopril
Captopril Tablets
Carbimazole Tablets
Carvedilol
Cefpodoxime Oral Suspension
Ceftazidime for Injection
Celecoxib
Activated Charcoal
Chlorpheniramine Tablets
Chlorthalidone
Chlorthalidone Tablets
Clemastine Tablets
Clindamycin Capsules
Clomipramine Capsules
Clonidine Tablets
Codeine Syrup
Codeine Tablets
Colchicine
Colchicine Tablets
Croscarmellose Sodium
Cyclizine Tablets
Cyclophosphamide
Cyclophosphamide Tablets
Cyclosporine Eye Drops
Cyproheptadine Tablets
Dalteparin Sodium
Dapsone
Deferasirox
Dexchlorpheniramine Maleate
Dextran 40 Infusion
Dextran 70 Infusion
Dicyclomine Tablets
Didanosine Gastro-resistant Capsules
Didanosine Tablets
Diethylcarbamazine Tablets
Diphenhydramine Capsules
Disulfiram
Disulfiram Tablets
Dobutamine Hydrochloride
Docetaxel Trihydrate
Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate Tablets
Donepezil Hydrochloride
Dothiepin Hydrochloride
Doxepin Capsules
Doxorubicin Hydrochloride
Doxorubicin Injection
Doxycycline Capsules
Dydrogesterone
Dydrogesterone Tablets
Econazole Cream
Efavirenz Tablets
Enoxaparin Sodium
Ergotamine Tablets
Esmolol Hydrochloride
Esomeprazole Gastro-resistant Tablets
Ethacrynic Acid Tablets
Ethanol
Ethanol (95 Per Cent)
Ethinyloestradiol Tablets
Ethosuximide
Fingolimod Hydrochloride
Flavoxate Tablets
Fludarabine Phosphate
Fludarabine Phosphate Injection
Fludrocortisone Tablets
Fluphenazine Tablets
Flurbiprofen Tablets
Framycetin Sulphate
Glipizide Tablets
Griseofulvin
Griseofulvin Tablets
Haloperidol Tablets
Heparin sodium
Hyoscine Butylbromide Tablets
Imatinib Mesylate
Imipenem and Cilastatin Injection
Indinavir Sulphate
Indinavir Capsules
Irinotecan Hydrochloride Trihydrate
Iron Dextran Injection
Isopropyl Alcohol
Isopropyl Rubbing Alcohol
Labetalol Hydrochloride
Labetalol Injection
Labetalol Tablets
Lamivudine and Tenofovir Tablets
Lamivudine and Zidovudine Tablets
Lamivudine Tablets
Lapatinib Ditosylate
Lapatinib Tablets
Levamisole Injection
Levetiracetam
Levofloxacin Hemihydrate
Levofloxacin Infusion
Levonorgestrel
Levonorgestrel and Ethinyloestradiol Tablets
Lincomycin Hydrochloride

Lincomycin Capsules
Lorazepam
Lorazepam Injection
Lorazepam Tablets
Luliconazole
Luliconazole Lotion
Magnesium Hydroxide
Maltodextrin
Marbofloxacin Injection
Mebendazole
Mebendazole Tablets
Meclizine Tablets
Megestrol Tablets
Menotropin
Menotropin for Injection
Methadone Tablets
Methyldopa Tablets
Methylparaben
Metoclopramide Tablets
Miconazole Cream
Mitomycin Injection
Montelukast Granules
Nabumetone Tablets
Nalidixic Acid Tablets
Natamycin
Nitrofurantoin
Nitrofurantoin Tablets
Norgestrel and Ethinyloestradiol Tablets
Oestradiol Injection
Olanzapine Tablets
Omeprazole and Domperidone Capsules
Omeprazole Gastro-resistant Capsules
Ondansetron Oral Solution
Ondansetron Tablets
Oxazepam Tablets
Oxprenolol Tablets
Oxygen
Oxygen 93 Per Cent
Oxytetracycline Capsules
Oxytetracycline Dihydrate
Oxytetracycline Hydrochloride
Oxytetracycline Hydrochloride Injection
Oxytetracycline Injection
Pantoprazole Gastro-resistant and Domperidone Prolonged-release Capsules
Pantoprazole Gastro-resistant Tablets
Paracetamol Oral Suspension
Paracetamol Paediatric Oral Suspension
Paracetamol Tablets
Penicillamine Tablets
Perphenazine Tablets
Phenindione Tablets
Phenobarbitone Tablets
Phenoxyethanol
Phenytoin sodium
Pindolol Tablets
Polyethylene Glycol 4000
Pravastatin Tablets
Pregabalin
Procainamide Tablets
Prochlorperazine Tablets
Procyclidine Tablets
Propylparaben
Propylthiouracil Tablets
Protriptyline Tablets
Pseudoephedrine Tablets
Pyridostigmine Tablets
Pyrimethamine Tablets
Quiniodochlor
Rabeprazole Gastro-resistant and Itopride Prolonged-release Capsules
Rabeprazole Gastro-resistant Tablets
Ranitidine Hydrochloride
Ranitidine Injection
Ranitidine Oral Solution
Ranitidine Tablets
Remdesivir Injection
Selegiline Tablets
Silver Sulphadiazine
Compound Sodium Lactate Injection
Sodium Phosphate
Sodium Starch Glycolate (Type A)
Sodium Valproate Gastro-resistant Tablets
Sodium Valproate Injection
Streptokinase Injection
Sterile Water for Injections
Talc
Tamoxifen Citrate
Tamoxifen Tablets
Tenofovir Alafenamide Fumarate
Tenofovir Disoproxil Fumarate
Testosterone Propionate
Theophylline
Theophylline Injection
Theophylline Prolonged-release Tablets
Thiocolchicoside
Thiomersal
Thiotepa
Thiotepa Injection
Thyroxine Sodium
Ticagrelor
Ticagrelor Tablets
Tigecycline
Tigecycline Injection
Timolol Tablets
Tiotropium Bromide Monohydrate
Tolazamide Tablets
Topiramate
Torsemide
Torsemide Tablets
Tranexamic Acid
Tranexamic Acid Injection
Tranexamic Acid Tablets
Triamcinolone Tablets
Triamterene Capsules
Trifluoperazine Tablets
Triflupromazine Tablets
Trimethoprim and Sulphamethoxazole Tablets
Trimethoprim Tablets
Triprolidine Tablets
Urokinase
Ursodeoxycholic Acid Tablets
Vancomycin Hydrochloride
Voriconazole
Warfarin Tablets
Zolmitriptan

Vitamins, Minerals, Amino Acids, Fatty Acids etc.

Ascorbic Acid
Ascorbic Acid Injection
Ascorbic Acid Tablets
Calcium Folinate Injection
Calcium Lactate
Calcium Lactate Tablets
Calcium Pantothenate
Ferrous Ascorbate
Folic Acid Tablets
Glycerin
Phytomenadione Injection
Pyridoxine Tablets
Riboflavin
Riboflavin Sodium Phosphate
Riboflavin Tablets
Thiamine Mononitrate
Thiamine Tablets

Phytopharmaceuticals

Aegle marmelos PPI
Andrographis paniculata PPI
Curcuma longa PPI
Gymnema sylvestre PPI
Lawsonia inermis PPI
Phyllanthus amarus PPI
Silybum marianum Dry Extract PPI

Herbs and Herbal Product

Acacia
Ajwain
Amalaki
Amaltas
Amarbel
Amla Juice Powder
Amra
Anantmula
Anise Oil
Arachis Oil
Arjuna
Arjuna Dry Extract
Artemisia
Ashwagandha
Ashwagandha Dry Extract
Asoka
Bakuci
Bala
Basil Oil (Methyl Chavicol Type)
Bassant
Bassant Dry Extract
Belladonna Leaf
Belladonna Dry Extract
Belladonna Dry Extract Tablets
Belladonna Soft Extract
Belladonna Tincture
Bhibhitaki Aqueous Extract
Bhringraj
Bhuiamla Dry Extract
Bimi
Black Pepper Oil
Brahmi
Brahmi Extract
Caraway Oil
Castor Oil (Refined)
Castor Oil (Virgin)
Citronella Oil (Java Type)
Clove Bud Oil
Coconut Oil
Dill Oil
Green Coffee Bean Extract
Nutmeg Oil
Shellac

Vaccines and Immunosera for Human Use

General Requirements

Vaccines
Antisera

Monographs

Adsorbed Diphtheria, Tetanus and Hepatitis B (rDNA) Vaccine
Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Haemophilus influenzae Type b Conjugate Vaccine
Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component), Inactivated poliomyelitis Vaccine and Haemophilus influenzae Type b Conjugate Vaccine
Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Inactivated poliomyelitis Vaccine
Adsorbed Pertussis (Acellular Component) Vaccine
Adsorbed Pertussis (Acellular; Co-purified) Vaccine
Diphtheria and Tetanus vaccine (Adsorbed)
Diphtheria and Tetanus vaccine (Adsorbed) for adults and adolescents
Diphtheria, Tetanus and Pertussis vaccine (Adsorbed)
Diphtheria, Tetanus, Pertussis (Whole Cell), Hepatitis B (rDNA) and Haemophilus influenzae Type b Conjugate Vaccine (Adsorbed)
Diphtheria, Tetanus, Pertussis (Whole Cell) and Hepatitis B (rDNA) Vaccine (Adsorbed)
Diphtheria, Tetanus, Pertussis (Whole Cell) and Haemophilus influenzae Type b Conjugate Vaccine (Adsorbed)
Diphtheria Vaccine (Adsorbed).
Diphtheria vaccine (Adsorbed) for adults and adolescents
Haemophilus influenza Type b Conjugate Vaccine
Hepatitis B Vaccine (rDNA).
Hepatitis A (Inactivated) Vaccine (Adsorbed)
Inactivated Hepatitis B Vaccine
Inactivated Influenza Vaccine (Split Virion)
Inactivated Influenza Vaccine (Surface Antigen)
Inactivated Influenza Vaccine (Whole Virion)
Influenza Vaccine (Human, Live Attenuated)

Japanese Encephalitis Vaccine (Human)
Japanese Encephalitis Live Vaccine (Human)
Japanese Encephalitis vaccine Inactivated (Adsorbed, Human)
Measles and Rubella Vaccine (Live)
Measles vaccine (Live)
Measles, Mumps and Rubella Vaccine (Live)
Group A Meningococcal Conjugate Vaccine
Meningococcal Polysaccharide Vaccine
Mumps Vaccine (Live)
Plague Vaccine
Pneumococcal Polysaccharide conjugate Vaccine (Adsorbed)
Poliomyelitis Vaccine, Live (Oral)
Rabies Vaccine, Human.
Rotavirus vaccine (Live attenuated, Oral)
Rubella vaccine (Live)
Scorpion Venom Antiserum
Tetanus Vaccine (Adsorbed)
Tick-borne Encephalitis Vaccine (Inactivated)
Typhoid Polysaccharide Vaccine
Typhoid Paratyphoid A Vaccine
Typhoid Vi Conjugate Vaccine
Typhus Vaccine
Varicella Vaccine, Live
Yellow fever Vaccine

Blood and Blood Related Products

Anti-A Blood Grouping Serum
Anti-B Blood Grouping Serum
Anti-AB Blood Grouping Serum
Anti-D (IgM) Monoclonal Blood Grouping Reagent
Anti Human Globulin (AHG) serum
Anti-D (Rho) Immunoglobulin
Anti-D Immunoglobulin for Intravenous Use
Blood Grouping Lectins Anti-A1
Blood Grouping Lectins Anti-H
Dried Human Anti-haemophilic Fraction
Fibrin Sealant Kit
Human Albumin
Human Normal Immunoglobulin for Intravenous use
Human Prothrombin Complex

Biotechnology Derived Therapeutic Products

Filgrastim Concentrated Solution
Follicle Stimulating Hormone
Follicle stimulating Hormone Concentrated Solution
Follicle Stimulating Hormone Injection
Pegfilgrastim
Recombinant Streptokinase Bulk Solution

Allergen Products

Allergen Products

Veterinary Monographs

Amprolium Hydrochloride and Ethopabate Premix
Amprolium, Ethopabate and Sulphaquinoxaline Premix
Decoquinate Premix
Dimetridazole Premix
Furazolidone Premix
Levamisole Injection
Lincomycin Premix
Oxyclozanide Primix
Oxytetracycline Hydrochloride Injection
Pentobarbitone Injection
Thiabendazole Premix
Infectious Bursal Disease Vaccine, Live
Peste Des Petits Ruminants Vaccine, Live

Change of Titles

General Chapters

From '2.4.42 Inductively Coupled Plasma Mass Spectrometry' to '2.4.42 Inductively Coupled Plasma Spectrometry'

From '2.8.2 Assay of Human Anti-D Immunoglobulin Method A' to '2.8.2 Assay of Human Anti-D Immunoglobulin'

Monographs

From 'Dextran 40 Injection' to 'Dextran 40 Infusion'

From 'Dextran 70 Injection' to 'Dextran 70 Infusion'

From 'Sodium Starch Glycollate' to 'Sodium Starch Glycolate (Type A)'

Monographs shifted from Chemical to Vitamins, Minerals, Amino Acids, Fatty Acids etc.

Alfacalcidol
Arginine
Ascorbic Acid
Ascorbic Acid Injection
Ascorbic Acid Tablets
Calcipotriol Anhydrous
Calcipotriol Ointment
Calcitriol
Calcitriol Capsules
Calcium Carbonate
Calcium Carbonate Tablets
Calcium Chloride
Calcium Chloride Injection
Calcium Folinate
Calcium Folinate Injection
Calcium Gluconate
Calcium Gluconate Injection
Calcium Gluconate Tablets
Calcium Lactate
Calcium Lactate Tablets
Calcium Levulinate
Calcium Levulinate Injection
Calcium Pantothenate
Calcium Pantothenate Tablets
Dibasic Calcium Phosphate
Tribasic Calcium Phosphate
Calcium and Vitamin D_3 Tablets
Cholecalciferol
Cholecalciferol Injection
Cholecalciferol Tablets
Cholecalciferol Concentrate (Powder Form)
Citric Acid
Citric acid monohydrate
Cyanocobalamin
Cyanocobalamin Injection

Ergocalciferol
Ergocalciferol Tablets
Ferrous Ascorbate
Ferrous Ascorbate and Folic Acid Suspension
Ferrous Ascorbate and Folic Acid Tablets
Ferrous Fumarate
Ferrous Fumarate Tablets
Ferrous Gluconate
Ferrous Gluconate Tablets
Ferrous Sulphate
Dried Ferrous Sulphate
Ferrous Sulphate Tablets
Folic Acid
Folic Acid Tablets
Folic Acid and Methylcobalamin Tablets
Glycine
Iron and Ammonium Citrate
Iron and Folic Acid Syrup
Iron and Folic Acid Tablets
Levocarnitine
Levocarnitine Tablets
Heavy Magnesium Carbonate
Light Magnesium Carbonate
Magnesium Chloride
Heavy Magnesium Oxide
Light Magnesium Oxide
Magnesium Sulphate
Magnesium Sulphate Injection
Magnesium Trisilicate
Methylcobalamin
Nicotinamide
Nicotinamide Tablets
Nicotinic Acid
Nicotinic Acid Tablets
D-Panthenol
Pepsin
Phytomenadione
Phytomenadione Injection
Potassium Chloride
Potassium Chloride for Injection
Potassium Citrate
Potassium Iodide
Potassium Sorbate
Pyridoxine Hydrochloride
Pyridoxine Tablets
Riboflavin
Riboflavin Sodium Phosphate
Riboflavin Tablets
Sodium Citrate
Thiamine Hydrochloride
Thiamine Injection
Thiamine Mononitrate
Thiamine Tablets
Tocopherol Acetate
Vitamin A Capsules
Vitamin A Concentrated Oil
Vitamin A Concentrate Powder
Water- miscible Vitamin A Concentrate
Vitamin A Pediatric Oral Solution
Vitamin A and D Capsules
Concentrated Vitamin D Solution
Concentrated Vitamin A and D Solution
Zinc Chloride
Zinc Chloride Injection
Zinc Sulphate
Zinc Sulphate Monohydrate
Zinc Sulphate Oral Solution
Zinc Sulphate Dispersible Tablets

Monographs shifted from Herbs and Herbal Products to Phytopharmaceuticals

Bael
Bhuiamla
Gudmar
Haridra
Henna
Kalmegh
Milk Thistle Dry Extract

Omissions

General Chapters

2.8.3 Assay of Human Anti-D Immunoglobulin Methods B and C

Monographs on Drug substances, Dosage forms and Pharmaceutical aids

Lorcaserin Hydrochloride Hemihydrate
Lorcaserin Hydrochloride Tablets

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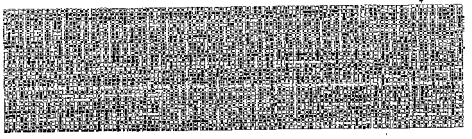

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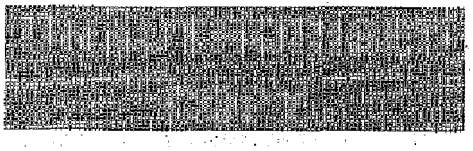

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General Notices

General Statements

The General Notices provide the basic guidelines for the interpretation and application of the standards, tests, assays, and other specifications of the Indian Pharmacopoeia (IP), as well as to the statements made in the monographs and other texts of the Pharmacopoeia.

A monograph is to be constructed in accordance with any general monograph or notice or any appendix, note or other explanatory material that is contained in this Pharmacopoeia and that is applicable to that monograph. All statements contained in the monograph, except where a specific general notice indicates otherwise and with the exceptions given hereafter, constitute standards for the official articles. An article is not of pharmacopoeial quality unless it complies with all of the requirements stated.

Exceptions to the General Notices do exist, and where they do, the wording in the individual monograph or an appendix takes precedence and specifically indicates directions or the intent. Thus, the specific wording of standards, tests, assays and other specifications is binding wherever deviations from the General Notices exist. Likewise, where there is no specific mention to the contrary, the General Notices apply.

Name. The full name or title of this book, including addenda thereto, is Indian Pharmacopoeia 2022, abbreviated to IP 2022. In the texts, the term "Pharmacopoeia" or "IP" without qualification means the Indian Pharmacopoeia 2022 and any amendments and thereto.

Official and Official Articles. The word 'official' wherever used in this Pharmacopoeia or with reference thereto, is synonymous with 'pharmacopoeial', with 'IP' and with 'compendial'. The designation IP in conjunction with the official title on the label of an article is an indication that the article purports to comply with IP standards.

The following terms are used where the articles for which monographs are provided are to be distinguished.

An official substance is a single drug or a drug entity or a pharmaceutical aid for which the monograph title includes no indication of the nature of a dosage form.

An official preparation is a drug product (dosage form) and is the finished or partially finished preparation or product of one or more official substances formulated for use on the patient.

An article is an item for which a monograph is provided, whether an official substance or an official preparation.

Official Standards. The requirements stated in the monographs apply to articles that are intended for medicinal use but not necessarily to articles that may be sold under the same name for other purposes.

An article is not of Pharmacopoeial quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product.

Pharmacopoeial requirements for articles used in veterinary medicine are established on the same basis as those used in human medicine. It should be noted that no requirement in the pharmacopoeia can be taken in isolation. A valid interpretation of any particular requirement depends upon it being read in context of the monograph as a whole, the specified method of analysis, the relevant General Notices and where appropriate the General Monographs.

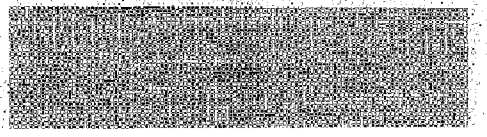
Where a preparation that is the subject of a monograph in the Indian Pharmacopoeia is supplied for use in veterinary medicine, the standards of Indian Pharmacopoeia apply unless otherwise justified and authorized.

The active pharmaceutical ingredients (drug substances), excipients (pharmaceutical aids), pharmaceutical preparations (dosage forms) and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses). It may be noted, however, that in the event of doubt of interpretation in any text of Veterinary monographs of IP, Indian Pharmacopoeia Commission (IPC) should be consulted.

The requirements given in the monographs are not framed to provide against all possible impurities, contaminants or adulterants; they provide appropriate limitation of potential impurities only.

A preparation must comply with the requirements specified, throughout its shelf-life assigned to it by the manufacturer. For opened or broached containers, the maximum period of validity for use will be as may be stated in the individual monograph. Nevertheless, the responsibility for assigning the period of validity shall be with the manufacturer.

Added Substances. An official substance, as distinguished from an official preparation, contains no added substances except when specifically permitted in the individual monograph. Unless otherwise specified in the individual monograph, or elsewhere in the General Notices, suitable substances may be added to an official preparation to enhance its stability, preserve its properties, usefulness or elegance, or to facilitate its preparation. Such auxiliary substances shall be harmless in the amounts used, shall not exceed the minimum quantity required to provide their intended effect, shall not impair the therapeutic efficacy or the bioavailability or safety of the preparation and shall not interfere with any of the tests and assays prescribed for determining compliance with the official



standards. Particular care should be taken to ensure that such substances are free from harmful organisms. The freedom to the manufacturers to add auxiliary substances imposes on them the responsibility of satisfying the licensing authorities on the purpose of the addition and the innocuity of such substances. No substance shall be added to conceal any defect or damage or deficiency in the substance or formulation.

Alternative Methods. The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. Automated procedures utilising the same basic chemistry as the test procedures given in the monograph may also be used to determine compliance. Such alternative or automated procedures must be validated and are subject to approval by the authority competent to authorised manufacturer of substance or product.

In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative and only the result obtained by the procedure given in this Pharmacopoeia is conclusive.

Meanings of Terms

Alcohol. The term "alcohol" without qualification means ethanol (95 per cent). Other dilutions of ethanol are indicated by the term "ethanol" or "alcohol" followed by a statement of the percentage by volume of ethanol (C_2H_6O) required.

Desiccator. A tightly-closed container of suitable size and design that maintains an atmosphere of low moisture content by means of silica gel or phosphorus pentoxide or other suitable desiccant.

Drying and ignition to constant weight. Two consecutive weighings after the drying or igniting operations do not differ by more than 0.5 mg, the second weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Ethanol. The term "ethanol" without qualification means anhydrous ethanol or absolute alcohol.

Filtration. Unless otherwise stated, filtration is the passing of a liquid through a suitable filter paper or equivalent device until the filtrate is clear.

Freshly prepared. Made not more than 24 hours before it is used.

Label. Any printed packing material, including package inserts that provide information on the article.

Negligible. A quantity not exceeding 0.50 mg.

Solution. Where the name of the solvent is not stated, "solution" implies a solution in water. The water used complies with the requirements of the monograph on Purified Water.

Temperature. The symbol ° used without qualification indicates the use of the Celsius thermometric scale.

Water. If the term is used without qualification it means Purified Water of the Pharmacopoeia. The term 'distilled water' indicates Purified Water prepared by distillation.

Water-bath. A bath of boiling water unless water at another temperature is indicated. Other methods of heating may be used provided the required temperature is approximately maintained but not exceeded.

Provisions Applicable To Monographs and Test Methods

Expression of Contents. Where the content of a substance is defined, the expression "per cent" is used according to circumstances with one of two meanings:

- per cent w/w (percentage, weight in weight) expressing the number of grams of substance in 100 grams of final product,
- per cent v/v (percentage, volume in volume) expressing the number of millilitres of substance in 100 millilitres of final product.

The expression "parts per million" refers to the weight in weight, unless otherwise stated.

Where the content of a substance is expressed in terms of the chemical formula for that substance an upper limit exceeding 100 per cent may be stated. Such an upper limit applies to the result of the assay calculated in terms of the equivalent content of the specified chemical formula. For example, the statement 'contains not less than 99.0 per cent and not more than 101.0 per cent of $C_7H_6O_2$' implies that the result of the assay is not less than 99.0 per cent and not more than 101.0 per cent, calculated in terms of the equivalent content of $C_7H_6O_2$.

Where the result of an assay or test is required to be calculated with reference to the dried, anhydrous, ignited substance, or the substance free from solvent, the determination of loss on drying, water content, loss on ignition, content of the specified solvent, respectively, is carried out by the method prescribed in the relevant test in the monograph.

Expression of Concentrations. The following expressions in addition to the ones given under Expression of Content are also used:

- per cent w/v (percentage, weight in volume) expressing the number of grams of substance in 100 millilitres of product,
- per cent v/v (percentage, volume in weight) expressing the number of millilitres of substance in 100 grams of product.

Usually, the strength of solutions of solids in liquids is expressed as percentage weight in volume, of liquids in liquids as percentage volume in volume, of solids in semi-solid bases (e.g. creams) and of gases in liquids as percentage weight in weight.

When the concentration of a solution is expressed as parts of dissolved substance in parts of solution, it means parts by weight (g) of a solid in parts by volume (ml) of the final solution, as parts by weight (g) of a gas in parts by weight (g) of the final solution.

When the concentration of a solution is expressed in molarity designated by the symbol M preceded by a number, it denotes the number of moles of the stated solute contained in sufficient Purified Water (unless otherwise stated) to produce 1 litre of solution.

Abbreviated Statements. Incomplete sentences are employed in parts of the monographs for directness and brevity (for example, Iodine Value. Not more than ..... ; Relative Density. .....to.....) Where the tests are abbreviated, it is to be understood that the test method referred to in brackets provides the method to be followed and that the values specified are the applicable limits.

Weights and Measures. The metric system of weights and measures is employed in the Pharmacopoeia. All measures are required to be graduated at 25° and all measurements in tests and assays, unless otherwise stated, are to be made at that temperature. Graduated glass apparatus used in analytical operations shall comply with the requirements stated in Chapter 2.1.6.

Monographs

General Monographs

General monographs on dosage forms include requirements of general application and apply to all preparations within the scope of the Introduction section of the general monograph, except where a preamble limits the application. The requirements are not necessarily comprehensive for a given specific preparation; additional requirements may sometimes be given in the individual monograph for it.

Production. Statements given under the heading Production relate to particular aspects of the manufacturing process and are not necessarily comprehensive. However, they are mandatory instructions to manufacturers. They may relate, for example, to source materials, to the manufacturing process and its validation and control, to any in-process testing that is to be carried out by the manufacturer on the final product either on selected batches or on each batch prior to release. All this cannot be verified on a sample of the final product by an independent analyst. It is for the licensing authority to verify that the instructions have been followed.

The absence of a section on Production does not imply that attention to features such as those given above is not required. An article described in a monograph of the Pharmacopoeia is to be manufactured in accordance with the principles of good manufacturing practice and in accordance with the requirements of the Drugs and Cosmetics Rules, 1945. The general principles applicable to the manufacture and quality assurance of drugs and preparations meant for human use apply equally to veterinary products as well.

Manufacture of Drug Products. The opening definitive statement in certain monographs for drug products is given in terms of the active ingredient(s) only. Any ingredient(s) other than those included in the statement, must comply with the general notice on Excipients and the product must conform to the Pharmacopoeial requirements.

Official preparations are prepared only from ingredients that comply with the requirements of the pharmacopoeial monographs for those individual ingredients for which monographs are provided.

Excipients. Any substance added in preparing an official preparation shall be innocuous, shall have no adverse influence in the therapeutic efficacy of the active ingredients and shall not interfere with the tests and assays of the Pharmacopoeia. Care should be taken to ensure that such substances are free from harmful organisms.

Individual Monographs

Drug products that are the subject of an individual monograph are also required to comply with the tests given in the general monographs.

Titles. The main title for a drug substance is the International Non-proprietary Name (INN) approved by the World Health Organization. Subsidiary names and synonyms have also been given in some cases; where included, they have the same significance as the main title.

The main titles of drug products are the ones commonly recognised in practice. Synonyms drawn from the full non-proprietary name of the active ingredient or ingredients have also been given. Where, however, a product contains one or the other of different salts of an active molecule, the main title is based on the full name of the active ingredient. For example, Chloroquine Phosphate Tablets and Chloroquine Sulphate Tablets.

Chemical Formulae. When the chemical structure of an official substance is known or generally accepted, the graphic and molecular formulae are normally given at the beginning of the monograph for information. This information refers to the chemically pure substance and is not to be regarded as an indication of the purity of the official material. Elsewhere, in statement of purity and strength and in descriptions of

processes of assay, it will be evident from the context that the formulae denote the chemically pure substances.

Where the absolute stereochemical configuration is specified, the International Union of Pure and Applied Chemistry (IUPAC) *R/S* and *E/Z* systems of designation have been used. If the substance is an enantiomer of unknown absolute stereochemistry, the sign of the optical rotation, as determined in the solvent and under the conditions specified in the monograph, has been attached to the systematic name. An indication of sign of rotation has also been given where this is incorporated in a trivial name that appears on an IUPAC preferred list.

Atomic and Molecular Weights. The atomic weight or molecular weight is shown, as and when appropriate at the top right hand corner of the monograph. The atomic and molecular weights and graphic formulae do not constitute analytical standards for the substances described.

Definition. The opening statement of a monograph is one that constitutes an official definition of the substance, preparation or other article that is the subject of the monograph. In certain monographs for pharmaceutical preparations the statement is given in terms of the principal ingredient(s).

In monographs on vegetable drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form.

Certain pharmaceutical substances and other articles are defined by reference to a particular method of manufacture. A statement that a substance or article is prepared or obtained by a certain method constitutes part of the official definition and implies that other methods are not permitted. A statement that a substance may be prepared or obtained by a certain method, however, indicates that this is one possible method and does not imply that other methods are not permissible.

Statement of content. The limits of content stated are those determined by the method described under Assay.

Category. The statement of category is provided for general information only and is indicative of the medical or pharmaceutical basis for recognition in the Pharmacopoeia. It generally represents an application of the best known pharmacological action of the article or of its active ingredient. The statement under the heading 'Category' are also subject to regulations under the D&C Act 1940 and rules thereunder. In the case of pharmaceutical aids it may indicate the more common usage of the article. The statement is not intended to limit in any way the choice or use of the article nor to indicate that it has no other activity or use.

Usual strength. The statement on the usual strength(s) of a preparation given in the individual monograph indicates the strength(s) usually marketed for information of the pharmacist and the medical practitioner. It does not imply that a strength other than the one(s) mentioned in the individual monograph meeting all the prescribed requirements cannot be manufactured and marketed with the approval of the appropriate authority.

Description. The statements under the heading Description are not to be interpreted in a strict sense and are not to be regarded as official requirements.

Solubility. Statements on solubility are given in Chapter 2.4.26 and are intended as information on the approximate solubility at a temperature between 15° and 30°, unless otherwise stated, and are not to be considered as official requirements. However, a test for solubility stated in a monograph constitutes part of the standards for the substance that is the subject of that monograph.

Residual solvents. The requirements, guidance and information on residual solvents for pharmaceutical use are given in the chapter entitled Residual Solvents (5.4).

All IP articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration and the solvents limited according to the principles defined and the requirements specified in Chapter 5.4. Residual Solvent, using the general methods presented therein or other suitable methods.

Test Methods

References to general methods of testing are indicated by test method numbers in brackets immediately after the heading of the test or at the end of the text.

Identification. The tests given under the heading Identification are not necessarily sufficient to establish absolute proof of identity. They provide a means of verifying that the identity of the material under examination is in accordance with the label on the container.

In certain monographs alternative series of identification tests are given, compliance with either one or the other set of tests is adequate to verify the identity of the article.

When tests for infrared absorption are applied to material extracted from formulated preparations, strict concordance with the specified reference spectrum may not always be possible, but nevertheless a close resemblance between the spectrum of the extracted material and the specified reference spectrum should be achieved.

Tests and Assays

The tests and assays are the official methods upon which the standards of the Pharmacopoeia depend. The requirements are not framed to take into account all possible impurities. It is

not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated. Material found to contain such an impurity is not of pharmacopoeial quality if the nature or amount of the impurity found is incompatible with good pharmaceutical practice.

Pharmacopoeial methods and limits should be used merely as compliance requirements and not as requirements to guarantee total quality assurance. Tests and assays are prescribed for the minimum sample available on which the attributes of the article should be measured. Assurance of quality must be ensured by the manufacturer by the use of statistically valid sampling and testing programmes.

Tests. Unless otherwise stated, the assays and tests are carried out at a temperature between 20° and 30°.

Where it is directed that an analytical operation is to be carried out 'in subdued light', precautions should be taken to avoid exposure to direct sunlight or other strong light. Where a procedure is directed to be performed 'protected from light' precautions should be taken to exclude actinic light by the use of low-actinic glassware, working in a dark room or similar procedures.

For preparations other than those of fixed strength, the quantity to be taken for a test or an assay is usually expressed in terms of the active ingredient. This means that the quantity of the active ingredient expected to be present and the quantity of the preparation to be taken are calculated from the strength stated on the label.

Other Tests. In the monographs on dosage forms and certain preparations, under the sub-heading 'Other tests' it is stated that the article complies with the tests stated under the general monograph of the relevant dosage form or preparation. Details of such tests are provided in the general monographs.

Limits. The limits given are based on data obtained in normal analytical practice. They take into account normal analytical errors, of acceptable variations in manufacture and of deterioration to an extent that is acceptable. No further tolerances are to be applied to the limits for determining whether or not the article under examination complies with the requirements of the monograph.

Quantities. Unless otherwise stated, the quantities to be taken for assays, limit tests and other tests are of the substance under examination.

In tests with numerical limits and assays, the quantity stated to be taken for testing is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result of analysis is calculated from this exact quantity. In tests where the limit is not numerical but usually depends upon comparison with the behaviour of a reference in the same conditions, the stated quantity is taken for testing. Reagents are used in the prescribed amounts.

Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision is plus or minus 5 units after the last figure stated. For example, 0.25 g is to be interpreted as 0.245 g to 0.255 g. For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero, e.g. 10.0 ml or 0.50 ml, the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; in other cases, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

The term 'transfer' is used generally to indicate a quantitative operation.

Apparatus. Measuring and weighing devices and other apparatus are described in the chapter entitled 'Apparatus for Tests and Assays'. A specification for a definite size or type of container or apparatus in a test or assay is given merely as a recommendation.

Unless otherwise stated, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base, commonly known as Nessler cylinders.

Reagents and Solutions. The reagents required for the tests and assays of the Pharmacopoeia are defined in the various chapters showing their nature, degree of purity and the strengths of the solutions to be made from them. The requirements set out are not intended to imply that the materials are suitable for use in the test concerned; reagents not covered by monographs in the pharmacopoeia shall not be claimed to be of IP quality.

The term 'analytical reagent grade of commerce' implies that the chemical is of a high degree of purity wherein the limits of various impurities are known. Where it is directed to use a 'general laboratory reagent grade of commerce' it is intended that a chemically pure grade material, not necessarily required to be tested for limiting or absence of certain impurities, is to be used.

Indicators. Where the use of an indicator solution is mentioned in an assay or test, approximately 0.1 ml of the solution shall be added, unless otherwise directed.

Reference Substances. Certain monographs require the use of a chemical reference substance or a biological reference preparation or a reference spectrum These are authentic specimens chosen and verified on the basis of their suitability for intended use as prescribed in the Pharmacopoeia and are not necessarily suitable in other circumstances.

IP Reference Substances, abbreviated to IPRS are issued by the Indian Pharmacopoeia Commission (IPC). They are the official standards to be used in cases of arbitration.

Biological Reference Substances, also abbreviated to IPRS and Standard Preparations of antibiotics are issued by

agencies authorised by the IPC. They are standardized against the International Standards and Reference Preparations established by the World Health Organization (WHO). The potency of these preparations is expressed in International Units.

Reference spectra are published by the IPC and they are accompanied by information concerning the conditions used for sample preparation and recording of the spectra.

Test Animals. The animal experiments are carried out in accordance with the provisions of 'The Prevention of Cruelty to Animals Act, 1960' and 'CPCSEA Guidelines' so as to prevent the infliction of unnecessary pain, suffering and prevention of cruelty to animals. Unless otherwise directed, animals used in a test or an assay shall be healthy and are drawn from a uniform stock, and have not previously been treated with any material that will interfere with the test or the assay.

Rounding Rules for Calculation of Results. The observed or calculated values should be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded up or down until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is 4 or smaller, it is eliminated and the preceding digit is left unchanged. If this digit is 5 to 9, it is eliminated and the preceding digit is increased by 1.

Table 1 – Illustration of Rounding Numerical values for comparison with Requirements

Pharmacopoeial Requirement	Unrounded Value (per cent)	Rounded Result (per cent)	Conforms
Assay: limit	97.96	98.0	Yes
≥ 98.0 per cent	97.92	97.9	No
	97.95	98.0	Yes
Assay: limit	101.55	101.6	No
≤ 101.5 per cent	101.46	101.5	Yes
	101.45	101.5	Yes
Limit: test	0.025	0.03	No
≤ 0.02 per cent	0.015	0.02	Yes
	0.027	0.03	No
Limit: test ≤ 3 ppm	3.5 ppm	4 ppm	No
	3.4 ppm	3 ppm	Yes
	2.5 ppm	3 ppm	Yes

Storage. Statements under the side-heading 'Storage' constitute non-mandatory advice. The articles of the Pharmacopoeia are to be stored under conditions that prevent contamination and, as far as possible, deterioration. Precautions that should be taken in relation to the effects of the atmosphere, moisture, heat and light are indicated, where appropriate, in the individual monograph.

Specific directions are given in some monographs with respect to the temperatures at which Pharmacopoeial articles should be stored, where it is considered that usage at a lower or higher temperature may produce undesirable results. The storage conditions are defined by the following terms:

— Store in a dry, well-ventilated place at a temperature not exceeding 30°

— Store in a refrigerator (2° to 8°). Do not freeze

— Store in a freezer (-2° to -18°)

— Store in a deep freezer (Below -18°)

Storage conditions not related to temperature are indicated in the following terms:

— Store protected from light

— Store protected from light and moisture

Where no specific storage directions or limitations are given in the monograph or in the D&C rules 1945 or by the manufacturer, it is to be understood that the storage conditions include protection from moisture, direct sunlight, freezing and excessive heat (any temperature above 40°).

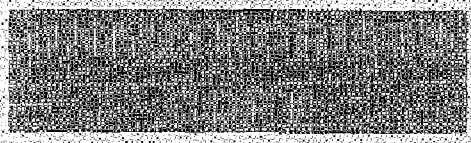
Storage Containers. The requirements, guidance and information on containers for pharmaceutical use are given in the chapter entitled Containers (6.2).

In general, an article should be packed in a well-closed container i.e. one that protects the contents from contamination by extraneous solids, liquids, moisture or vapours and from loss of the article under normal conditions of handling and storage and preserves the properties of the drug. Containers, unless otherwise specified, or of the nature such as capsule shall, foils of strips etc, shall allow examination of the contents inside. Closures used shall also of suitable properties and quality to protect the drug from any contamination and shall not be the source of contamination by themselves. Notices as may be needed in respect of Radiopharmaceuticals may also be incorporated.

Where, additionally, loss or deterioration of the article from effervescence, deliquescence or evaporation under normal conditions of storage is likely, the container must be capable of being tightly closed, and re-closed after use.

In certain cases, special requirements of pack have been indicated in some monographs under Storage, using expressions that have been defined in chapter 6.2.

Labelling. The labelling of drugs and pharmaceuticals is governed by the Drugs and Cosmetics Rules, 1945. The statements that are given in the monographs under the side-heading 'Labelling' are not comprehensive. Only those that are necessary to demonstrate compliance or otherwise with the monograph have been given and they are mandatory. For example, in the monograph on Betamethasone Sodium Tablets the labelling statement is "The label states the strength in terms of the equivalent amount of betamethasone".



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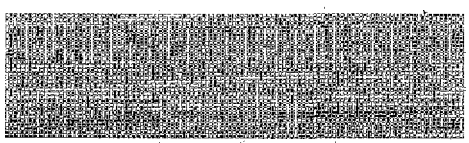

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2.1.1. Gas Detector Tubes

Gas detector tubes are cylindrical, sealed tubes consisting of an inert transparent material and constructed to allow the passage of gas. They contain reagents adsorbed onto inert substrates that are suitable for the visualisation of the substance to be detected and, if necessary, they also contain preliminary layers and/or adsorbent filters to eliminate substances that interfere with the substance to be detected. The layer of indicator contains either a single reagent for the detection of a given impurity or several reagents for the detection of several substances (monolayer tube or multilayer tube).

The test is carried out by passing the required volume of the gas under examination through the indicator tube. The length of the coloured layer or the intensity of a colour change on a graduated scale gives an indication of the impurities present.

The calibration of the detector tubes is verified according to the manufacturer's instructions.

Operating conditions

Examine according to the manufacturer's instructions or proceed as follows:

The gas supply is connected to a suitable pressure regulator and needle valve. Connect the flexible tubing fitted with a Y-piece to the valve and adjust the flow of gas under examination to purge the tubing to an appropriate flow (see Fig. 2.1.1-1). Prepare the indicator tube and fit to the metering pump. Connect the open end of the indicator tube to the short leg of the tubing and operate the pump by the appropriate number of strokes to pass a suitable volume of the gas under examination through the tube. Read the value corresponding to the length of the coloured layer or the intensity of the colour on the graduated scale. If a negative result is achieved, indicator tubes can be verified with a calibration gas containing the appropriate impurity.

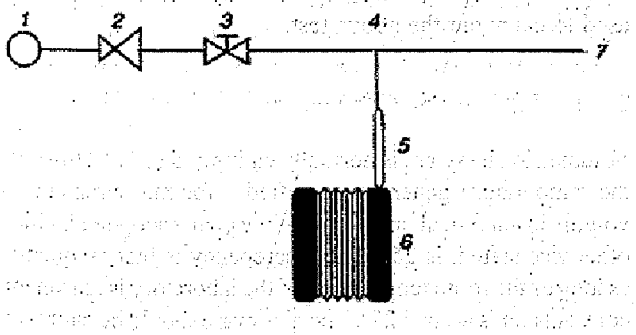


Fig. 2.1.1-1: Apparatus for Gas Detector Tubes

1. gas supply
2. pressure regulator
3. needle valve
4. "Y" piece
5. indicator tube
6. indicator tube pump
7. end open to atmosphere

In view of the wide variety of available compressor oils, it is necessary to verify the reactivity of the oil detector tubes for the oil used. Information on the reactivity for various oils is given in the leaflet supplied with the tube. If the oil used is not cited in the leaflet, the tube manufacturer must verify the reactivity and, if necessary, provide a tube specific for this oil.

Carbon dioxide detector tube: Sealed glass tube containing adsorbent filters and suitable supports for hydrazine and crystal violet indicators. The minimum value indicated is 100 ppm with a relative standard deviation of not more than ± 15 per cent.

Sulphur dioxide detector tube: Sealed glass tube containing adsorbent filters and suitable supports for the iodine and starch indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of not more than ± 15 per cent.

Oil detector tube: Sealed glass tube containing adsorbent filters and suitable supports for the sulphuric acid indicator. The minimum value indicated is 0.1 mg/m^3 with a relative standard deviation of not more than ± 30 per cent.

Nitrogen monoxide and nitrogen dioxide detector tube: Sealed glass tube containing adsorbent filters and suitable supports for an oxidising layer (*Cr (VI) salt*) and the diphenylbenzidine indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of not more than ± 15 per cent.

Carbon monoxide detector tube: Sealed glass tube containing adsorbent filters and suitable supports for di-iodine pentoxide, selenium dioxide and fuming sulphuric acid indicators. The minimum value indicated is 5 ppm or less, with a relative standard deviation of not more than ± 15 per cent.

Hydrogen sulphide detector tube: Sealed glass tube containing adsorbent filters and suitable supports for an appropriate lead salt indicator. The minimum value indicated is 1 ppm or less, with a relative standard deviation of not more than ± 10 per cent.

Water vapour detector tube: Sealed glass tube containing adsorbent filters and suitable supports for the magnesium perchlorate indicator. The minimum value indicated is 67 ppm or less, with a relative standard deviation of not more than ± 20 per cent.

2.1.2. Nessler Cylinders

Nessler cylinders which are used for comparative tests are matched tubes of clear, colourless glass with a uniform internal diameter and a flat, transparent base. They comply with IS4161:1967.

A column of the liquid is examined down the vertical axis of the tube against a white background , or if necessary, against a black background. The examination is carried out in diffused light.

It is assumed that tubes with an internal diameter of 16 mm will be used. Tubes with a larger internal diameter may be used instead but the volume of liquid examined must then be increased so that the depth of liquid in the tubes is not less than where the prescribed volume of liquid and tubes 16 mm in internal diameter are used.

2.1.3. Sieves

Sieves for pharmacopoeial testing are of wire mesh woven from brass, bronze, stainless steel or other suitable wire and are not coated or plated. The sieves shall be devoid of defects and damages. The wires are of uniform circular cross-section. There must be no reaction between the material of the sieves and the substance being sifted. Sieves conform to the specifications given in Table.

Table

Approximate sieve number*	Approximate per cent sieving area	Nominal mesh aperture size (mm)	Tolerance average aperture size (± mm)
4	55	4.0	0.136
8	48	2.0	0.07
10	46	1.7	0.06
12	44	1.4	0.05
16	41	1.0	0.03
		µm	± µm
22	37	710	25
25	36	600	21
30	38	500	18
36	36	425	15
44	38	355	13
60	37	250	13 (9.9)**
85	35	180	11 (7.6)
100	36	150	9.4 (6.6)
120	34	125	8.1 (5.8)
150	36	106	7.4 (5.2)
170	35	90	6.6 (4.6)
200	36	75	6.1 (4.1)
240	34	63	5.3 (3.7)
300	35	53	4.8 (3.4)
350	34	45	4.8 (3.1)

*Sieve number is the number of meshes in a length of 2.54 cm in each transverse direction parallel to the wires.

**Figures in parentheses refer to close tolerances; those without parentheses relate to full tolerances.

2.1.4. Thermometers

Unless otherwise specified, thermometers suitable for pharmacopoeial tests conform to Indian Standard 4825:1968 and are standardised in accordance with the Indian Standard 6274:1971, Method of Calibrating Liquid-in-Glass Thermometers. The thermometers are of the mercury-in-glass type and the column above the liquid is filled with nitrogen. They may be standardised for total immersion or for partial immersion. To the extent possible, each thermometer should be employed according to the conditions of immersion under which it was standardised. In the selection of a thermometer, it is essential to consider the conditions under which it is to be used.

2.1.5. Ultraviolet Ray Lamps

The viewing of thin-layer chromatograms is done with the aid of a source of ultraviolet light such as a mercury vapour in a quartz lamp. A suitable filter may be fitted to eliminate the visible part of the spectrum emitted by the lamp. Where the monograph prescribes viewing under ultra-violet light of wavelength 254 nm or 365 nm, an instrument consisting of a mercury vapour lamp and a filter which gives an emission band with maximum intensity at about 254 nm or 365 nm should be used. The lamp should be capable of revealing without doubt a standard spot of sodium salicylate with a diameter of about 5 mm on a chromatographic plate coated with *silica gel G*. For this purpose the following test may be carried out.

Apply to a plate coated with *silica gel G*, 5 µl of a 0.04 per cent w/v solution of *sodium salicylate* in *ethanol (95 per cent)* for lamps of maximum output at about 254 nm and 5 µl of a 0.2 per cent w/v solution of sodium salicylate in *ethanol (95 per cent)* for lamps of maximum output at about 365 nm. Examine the spot in a position normal to the radiation. The distance between the lamp and the plate under examination used in a pharmacopoeial test should not exceed the distance used to carry out the above test.

2.1.6. Volumetric Glassware

Volumetric glassware is normally calibrated at 27°. However, the temperature generally specified for measurements of volume in analytical operations of the pharmacopoeial, unless otherwise stated, is 25°. The discrepancy is inconsequential as long as the room temperature in the laboratory is reasonably constant and is around 27°. The glassware should be calibrated periodically as per IS 4787:2010.

Pharmacopoeial assays and tests involving volumetric measurements require the use of accurately calibrated glassware. Volumetric apparatus must be suitably designed to assure accuracy. The design, construction and capacity of

Table 1

Volumetric Flasks: IS 915:2012									
Nominal capacity, ml	5	10	20	25	50	100	250	500	1000
Tolerance, ± ml									
Class A	0.025	0.025	0.04	0.04	0.06	0.1	0.15	0.25	0.4
Class B	0.05	0.05	0.08	0.08	0.12	0.2	0.3	0.5	0.8
One-Mark Pipettes: IS 1117 :1975									
Nominal capacity, ml	1	2	5	10	20	25	50	100	
Tolerance, ± ml									
Class A	0.007	0.01	0.015	0.02	0.03	0.03	0.05	0.08	
Class B	0.015	0.02	0.03	0.04	0.06	0.06	0.1	0.16	
Graduated Pipettes: IS 835:2007									
Nominal capacity, ml	1	2	5	10	25				
Subdivision, ml	0.01	0.02	0.05	0.1	0.2				
Tolerance, ± ml									
Class A	0.007	0.01	0.03	0.05	0.1				
Class B	0.01	0.02	0.05	0.1	0.2				
Burettes: IS 1997:2008									
Nominal capacity, ml	10	25	50	100					
Subdivision, ml	0.05	0.05	0.1	0.2					
Tolerance, ± ml									
Class A	0.03	0.03	0.05	0.1					
Class B	0.05	0.05	0.1	0.2					

volumetric glassware should be in accordance with those laid down by the Bureau of Indian Standards. There are two grades of apparatus available, designated as Class A and Class B. Class B apparatus may be employed in routine work; Class A is intended for use in work of the highest accuracy. The tolerances on capacity for volumetric flasks, pipettes and burettes, as laid down in the relevant Indian Standards, are set out in Table 1.

Where it is directed that a quantity be 'accurately measured', the apparatus must be chosen and used with care. A burette should be of such size that the titrant volume represents not less than 30 per cent of the nominal volume. Where less than 10 ml of titrant is to be measured, a 10 ml microburette is generally required.

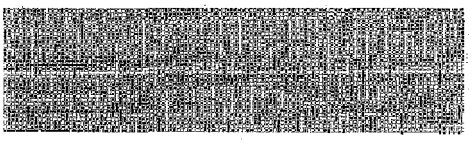
2.1.7. Weights and Balances

Pharmacopoeial assays and tests require the use of analytical balances that vary in capacity, sensitivity and reproducibility. The accuracy needed for a weighing indicates the type of balance. Where substances are to be 'accurately weighed', the weighing is to be performed so as to limit the error to not more than 0.1 per cent. For example, a quantity of 50 mg is to be weighed so that the error does not exceed 0.05 mg. A balance should be chosen such that the value of three times the standard deviation of the reproducibility of the balance, divided by the amount to be weighed, does not exceed 0.001.

Balances should be calibrated periodically against absolute standard weights.

Class 1.1 weights are used for calibration of low-capacity, high sensitivity balances. They are available in various denominations from 1 to 400 mg. The tolerance for any denomination in this class is 5 µg. They are recommended for calibration of balances for using optical or electrical methods for accurately weighing quantities below 20 mg.

Class 1 weights are designated as high-precision standards for calibration. They may be used for weighing accurately quantities below 20 mg.



Class 2 weights are used as working standards for calibration, built-in weights for analytical balances, and laboratory weights for routine analytical work.

Class 2 and class 4 weights are used with moderate-precision laboratory balances.

2.1.8. Continuous Extraction of Drugs

Where continuous extraction of a drug or any other substance is recommended in the monograph, percolation should be carried out. The process consists of percolating it with a suitable solvent at a temperature approximately that of the boiling-point of the solvent. Any apparatus that permits the uniform percolation of the drug and the continuous flow of the vapour of the solvent around the percolator may be used. The type commonly known as the soxhlet apparatus is suitable for this purpose.

A simple apparatus is shown in Fig 2.1.8-1. A is an outer tube of stout glass; the wider part is about 18 cm in length and has an internal diameter of 4.8 to 5 cm; the lower end C is about 5 cm in length and has an external diameter of about 1.6 cm. B is a straight glass tube open at both ends, about 9 cm in length and having an external diameter of about 3.8 cm; over its lower flanged end is tied firmly a piece of calico or other suitable material. D is a glass coil, which supports the margin of the tube B and prevents it from resting in contact with the outer tube A. The lower end C of the outer tube A is fitted to the distilling flask E, in which a suitable quantity of the solvent has been placed. The substance to be extracted, previously moistened with the solvent or subjected to any preliminary treatment required, is introduced into the inner tube B, which is supported so that the percolate drops into the outer tube. A pad of cotton wool G is placed on the top of the drug, the inner tube is lowered into position and outer tube connected by means of a suitable cork with the tube of a reflux condenser F. The flask is heated and the extraction continued as directed.

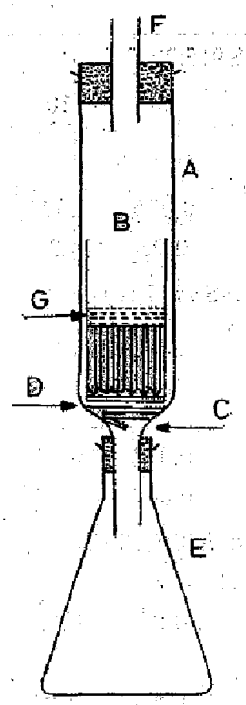


Fig. 2.1.8-1:Apparatus for continuous extraction of Drugs

2.2. BIOLOGICAL METHODS

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2.2.1. Abnormal Toxicity

General test. Inject intravenously into each of five healthy mice, weighing 17 g to 22 g, the quantity of the substance under examination in 0.5 ml of *water for injections* or of sterile *normal saline solution*, over a period of 15 to 30 seconds, unless otherwise stated.

The substance passes the test if none of the mice dies within 24 hours or within the time specified in the individual monograph. If more than one animal dies, the preparation fails the test. If one of the animals dies, repeat the test. The substance passes the test if none of the animals in the second group die within the time interval specified.

For antisera and vaccines. Unless otherwise prescribed in the individual monograph inject intra-peritoneally one human dose but not more than 1.0 ml into each of five healthy mice, weighing 17 g to 22 g, and one human dose but not more than 5.0 ml into each of two healthy guinea pigs weighing 250 g to 350 g. The human dose is that stated on the label or in the accompanying information leaflet of the preparation under examination.

The preparation passes the test if none of the animals dies or shows signs of ill-health in 7 days following the injection. If more than one animal dies, the preparation fails the test. If one of the animals die or show signs of ill health, repeat the test. The preparation passes the test if none of the animals in the second group dies or show signs of ill health in the time interval specified.

2.2.2. Effectiveness of Antimicrobial Preservatives

NOTE—The test for effectiveness of antimicrobial preservatives shall be demonstrated during development of pharmaceutical preparation and during the commercial manufacturing. The test is not intended to be used for routine control purpose.

The efficacy of antimicrobial preservation of a pharmaceutical preparation on its own or, if necessary, with the addition of a suitable preservative has to be ascertained during the development of the product. The primary purpose of adding antimicrobial preservatives to dosage forms is to prevent adverse effects arising from contamination by microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process. However, antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing procedures. There may be situations where a preservative system may have to be used to minimize proliferation of microorganisms in preparations that are not required to be sterile. It should be recognized that the presence of dead microorganisms or their metabolic by-products may cause adverse reactions in sensitized persons.

Any antimicrobial agent may show the protective properties of a preservative. However, for the protection of the consumer the concentration of the preservative shown to be effective in the final packaged product should be considerably below the concentrations of the preservative that may be toxic to human beings.

Antimicrobial efficacy test must be demonstrated for multiple dose parenteral, otic, nasal, opthalmic, oral and topical products made with aqueous bases or vehicles, the effectiveness of any added preservatives, during the shelf-lives of the preparations to ensure that the antimicrobial activity has not been impaired by storage. The tests apply only to the product in the original, unopened container in which it was supplied by the manufacturer.

The test consists of challenging the preparation in its final container with a prescribed inoculum of suitable microorganisms, storing the inoculated product at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples removed. The preservative properties of the product are considered adequate if, in the conditions of the test, there is a significant fall or no increase in the number of microorganisms in the inoculated preparation after storage for the times and at the temperatures prescribed.

The organisms specified for use in the tests are intended to be representative of those that might be expected to be found in the environment in which the preparation is manufactured, stored and used. However, they should be supplemented by other strains or species, especially those likely to be found in the conditions under a particular product is made or used, or that might offer a particular challenge to the type of product being tested. Single strain challenges (rather than mixed cultures) should be used throughout.

Precautions. Challenge tests should be conducted under conditions that prevent accidental contamination of the product during the test but the precautions taken to prevent contamination should not affect the survival of organisms in the product being examined.

Test organisms. The following test organisms are used in the test.

Candida albicans ATCC 10231*
Aspergillus brasiliensis ATCC 16404
Escherichia coli ATCC 8739
Pseudomonas aeruginosa ATCC 9027
Staphylococcus aureus ATCC 6538

*Instead of *Candida albicans*, *Zygosaccharomyces rouxii* NCYC 381; IP 2021.92 may be used for oral preparations containing high concentration of sugar.

In order to prevent any phenotypic changes in the strains used, the organisms used in the test should not be more than 5 passages made from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium.

NOTE— All the media used in the tests should be tested for growth promotion.

Preparation of inoculum. Grow each of the bacterial species separately in Casein soyabean digest agar and incubate them at 30° to 35° for 18 to 24 hours. Grow *Candida albicans* on Sabouraud dextrose agar and incubate at 20° to 25° for atleast 48 hours. Grow *Aspergillus brasiliensis* on Sabouraud dextrose agar at 20° to 25° for 5-7 days. After incubation, harvest the growth and resuspend each of the organisms separately in sterile saline to obtain a microbial count of $1x10^8$ CFU per ml. To suspend spores of *Aspergillus brasiliensis*, 0.05 per cent *polysorbate 80* may be added to the saline. Use suspension of these organisms within 2-4 hours. The suspension may be stored at 4° to 8° for a validated period of time.

Remove immediately a suitable sample from each suspension and determine the number of CFU per ml. in each suspension by pour plate method or filtration method. This value serves to determine the inoculum concentration and the baseline to use in the test.

Procedure. If sufficient volume (atleast 20 ml) of product is available in each container and the product container can be inoculated aseptically then the test can be conducted in five original containers of the product. If filled volume is less, or the container cannot be inoculated aseptically then transfer (atleast 20 ml) the product in each of five suitable sterile containers. Inoculate each container with one of the prepared and standardized inoculum in such a way that after inoculation the final concentration of the organisms remains between $1x10^5$ and $1x10^6$ CFU per ml and the volume of the inoculum does not exceed 1 per cent of the volume of the product. The initial concentration of the viable organisms in each test preparation is estimated based on the concentration of the microorganisms in each of the standardized inoculum as determined by the pour plate method or membrane filtration method.

Incubate the inoculated containers at room temperature. Determine the viable count by plate-count method at 7, 14, and 28 days subsequent to the inoculation. Record any changes observed in the appearance at these intervals. From the calculated concentration of CFU per ml present at the start of the test, calculate the percentage of reduction in CFU per ml for each organism at the stated test intervals and express the changes in terms of percentage of initial concentration.

Interpretation. The preservatives are considered to be effective if:

i) For parenteral, ophthalmic, sterile nasal and otic preparations: (a) the concentration of the viable bacteria are not more than 10 per cent of the initial concentration at 7 day and not more than 0.1 per cent of the initial concentration at 14 day and there is a further decrease in count at 28 day. (b) there is no increase in yeast and mold count at 7, 14 and 28 day from the initial count.

ii) For topical preparations made with aqueous base, non-sterile nasal preparation and emulsions including those applied to mucous membrane: (a) the concentration of the viable bacteria are not more than 1 per cent of the initial concentration at 14 days and there is a further decrease in count at 28 day. (b) there is no increase in yeast and mold count at 14 and 28 day from the initial count.

iii) For oral preparations : (a) the concentration of the viable bacteria are not more than 10 per cent of the initial concentration at 14 days and there is a further decrease in count at 28 day. (b) there is no increase in yeast and mold count at 14 and 28 days from the initial count.

2.2.3. Bacterial Endotoxins

The test for bacterial endotoxins (BET) measures the activity of bacterial endotoxins that may be present in the sample or on the article to which the test is applied using a lysate derived from the hemolymph cells or amoebocytes of the horseshoe crab, *Limulus polyphemus*. Other species of horseshoe crab namely *Tachypleus gigas*, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* also yield amoebocyte lysate having similar activity.

The addition of a solution containing endotoxins to a solution of the lysate produces turbidity or gelation of the mixture. However, addition of a chromogenic substrate to a solution of the lysate results in development of colour due to release of chromophore from the substrate upon activation by the endotoxin present in the solution. The rate of reaction depends on the activity of endotoxin, pH and temperature. The reaction requires the presence of certain bivalent cations, a clotting cascade enzyme system and clottable protein, all of which are provided by the lysate.

The following methods can be used to monitor the endotoxin activity in a product official in the Pharmacopoeia and to determine whether the product complies with the limit specified in the monograph.

Method A. Gel-Clot Limit Test Method

Method B. Semi-quantitative Gel-Clot Method

Method C. Kinetic Turbidimetric Method

Method D. Kinetic Chromogenic Method

Method E. End-Point Chromogenic Method

When a monograph includes a test for bacterial endotoxins without mentioning a method, the test is carried out by Method A. Any one of the other four methods may be employed as an alternative method provided it yields results of equivalent reliability with the preparation under examination.

The quantities of endotoxins are expressed in defined Endotoxin Units (EU). With the adoption of the second International Standard for endotoxin by the Expert Committee on Biological Standards of the World Health Organization, 1 EU = 1 IU.

The endotoxin limit for a given test preparation is calculated from the expression K/M, where M is the maximum dose administered to an adult (taken as 70 kg for this purpose) per kg per hour and K is the threshold pyrogenic dose of endotoxin per kg of body mass. The value of K is 5.0 EU per kg for parenteral preparations except those administered intrathecally, and is 0.2 EU per kg for preparations intended to be administered intrathecally.

For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175/V, where V is the maximum recommended dose in ml. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14/V. For formulations (anticancer products) administered on a per square meter of body surface, the formula is K/M, where K= 2.5 EU per kg and M is the (maximum dose/m^2/hour x 1.80 m^2)/70 kg.

The test should be carried out in a manner that avoids endotoxin contamination. If necessary, the containers should be treated to eliminate surface endotoxins that may be present by heating in an oven at 250° for 30 minutes provides more than 3 log reductions or by using a validated oven cycle or by any other means.

Before carrying out the test for endotoxins in the preparation under examination it is necessary to verify

(a) in the case of gel-clot methods, the sensitivity of the lysate;

(b) in the case of quantitative methods, the linearity of the standard curve;

(c) the absence of interfering factors, which inhibit or enhance the reaction or otherwise interfere with the test on the preparation under examination;

(d) the adequacy of the containers to resist adsorption of endotoxins.

Special Reagents

Endotoxin Reference Standard and Control Standard Endotoxin. The Endotoxin Reference Standard (ERS) is the freeze-dried, purified endotoxin of *Escherichia coli* 055:B5 is used as the Control Standard Endotoxin which is calibrated in Endotoxin Units (EU) by comparison with the International Standard.

The freeze-dried endotoxin should be reconstituted with *water BET* by mixing intermittently for 30 minutes using a vortex mixer. The concentrate should be stored in a refrigerator for not more than 28 days. Subsequent dilutions of the concentrate should be made by mixing vigorously for not less than 5 minutes before use. Each dilution should be mixed for not less than 1 minute before proceeding to make the next dilution.

A Control Standard Endotoxin (CSE) which is suitably standardised against the ERS may be used for routine bacterial endotoxin testing.

Lysate. A lysate of amoebocytes from either of the species of the horseshoe crab, *Limulus polyphemus*, *Tachypleus gigas*, *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda* reconstituted as stated on the label.

The species from which the lysate is obtained is stated on the label.

Water BET. Water BET is prepared by a suitable method that gives a negative result under the conditions prescribed in the test for bacterial endotoxins on the preparation under examination.

0.1 M Hydrochloric acid BET. Prepare from *hydrochloric acid* using *water BET*. After adjustment of the pH 6.0 to 8.0 with *0.1M sodium hydroxide BET* it gives a negative result under the conditions of the test.

0.1 M Sodium hydroxide BET. Prepare from *sodium hydroxide* using *water BET*. After adjustment of the pH to 6.0 to 8.0 with *0.1M hydrochloric acid BET* it gives a negative result under the conditions of the test.

***Tris*-chloride buffer pH 7.4 BET**. Dissolve 0.6057 g of *tris (hydroxymethyl) methylamine* in 30 ml of *water BET*, add 0.33 ml of *hydrochloric acid*, dilute to 100 ml with *water BET* and mix. It gives a negative result under the conditions of the test.

β-D-Glucan blocking buffer. β-D-Glucan blocking buffer is used to address the interference arising due to 1-3 β-D-Glucans during endotoxin assay. The buffer inhibits the Factor G pathway and effectively blocks reactions with 1-3 β-D-Glucans.

NOTE — Special reagents used in the test may use the suffix 'LAL', 'TAL' or 'CAL', as the case may be, to indicate the species of the horseshoe crab from which the amoebocyte lysate is derived. They have the same significance as the suffix 'BET'.

Gel-Clot Methods

Methods A and B depend on the formation of a firm gel when a solution containing bacterial endotoxins is incubated after mixing with the lysate. Method A is conducted as a limit test wherein both the replicate solutions of the preparation under examination must contain endotoxin in the concentration less than the endotoxin limit concentration specified in the individual monograph. Method B determines the endotoxin concentration semiquantitatively in the preparation under examination.

Sensitivity of the lysate. Confirm the labelled sensitivity of each new batch of lysate prior to use in the test using at least one vial of each batch of lysate. Prepare a series of dilutions of CSE to give concentrations of 2λ, λ, 0.5 λ and 0.25λ, where λ is the labelled sensitivity of the lysate in EU per ml. Perform the test as given under Method on these four standard concentrations in duplicate and include a negative control consisting of *water BET*. At least the final dilution in each series must give a negative result.

Dilution	Result
2λ	+
λ	+/–
0.5λ	+/–
0.25λ	–

+ = positive (gel clot present); – = negative (gel clot absent)

Calculate the average of the logarithms of the lowest concentration of endotoxin in each series of dilutions for which a positive result is found. The geometric mean end-point concentration is the measured sensitivity of the lysate in EU per ml, which is calculated using the following expression:

$$\text{Geometric mean end-point concentration} = \text{antilog}\left(\frac{\Sigma e}{f}\right)$$

where, Σe = sum of the log end-point concentrations of the series of dilutions used;

f = number of replicate test-tubes.

This average gives the estimated lysate sensitivity which must lie between 0.5λ and 2λ.

Test for interfering factors. The possibility of interference with the bacterial endotoxins test by certain factors should be borne in mind. For validation of the test results it must be demonstrated that the test preparation does not inhibit or enhance the reaction or otherwise interfere with the test. The validation must be repeated if the lysate vendor or the method of manufacture or the formulation of the sample is changed. Dilution of the test preparation with *water BET* is the easiest method for overcoming inhibition.

The allowable dilution level or Maximum Valid Dilution (MVD) is dependent on the concentration of the product, the endotoxin limit for the product and the lysate sensitivity. It is calculated by the following expression:

$$\text{MVD} = \frac{\text{Endotoxin limit} \times \text{Concentration of the test solution}^*}{\lambda}$$

where, λ is the labelled sensitivity of the lysate (EU/ml).

* Concentration of the test solution is expressed as mg/ml in case the endotoxin limit is specified by weight (EU/mg), or as Units/ml in case the endotoxin limit is specified by Unit (EU/Unit), or as 1.0 ml/ml in case the endotoxin limit is specified by volume (EU/ml).

Preparation of test solutions. Prepare replicates of solutions A to D as indicated in the table.

Solution	Final concentration of added CSE in the solution	Number of replicates
A	–	4
B	2λ	4
	λ	4
	0.5λ	4
	0.25λ	4
C	2λ	2
	λ	2
	0.5λ	2
	0.25λ	2
D	–	2

Solution A = Solution of the product at a dilution or at below MVD (test solution).

Solution B = Test solution spiked with indicated CSE concentrations (Positive Product Control; PPC).

Solution C = Standard solution with indicated CSE concentrations in *water BET*.

Solution D = *Water BET* (Negative Control; NC).

Method. Carry out the following procedure in receptacles such as tubes, vials or wells of micro-titre plates. Into each of the chosen receptacle, add an appropriate volume of negative control (NC), standard CSE solutions in *water BET*, test solution and positive product control (PPC). At intervals that will permit the reading of each result, add to each receptacle an equal volume of the appropriately constituted lysate unless single test vials are used. Mix the sample-lysate mixture gently and place in an incubating device such as a water-bath or a heating block, accurately recording the time at which the receptacles are so placed. Incubate each receptacle at 37° ± 1° undisturbed for 60 ± 2 minutes. Remove the receptacles and examine the contents carefully. A positive reaction is characterised by the formation of a firm gel that retains its integrity when inverted through 180° in one smooth motion. Record this result as positive (+). A negative result is

characterised by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (–). Handle the receptacles with care to avoid subjecting them to unwanted vibrations as false negative observations may result.

Calculate the geometric mean end-point concentration of solutions of series B and C by using the formula described under Sensitivity of the lysate.

Calculation and interpretation of results. The test for interfering factors is valid if

(a) solutions of series A and D give negative results;

(b) the results obtained with solutions of series C confirm the labelled sensitivity of the lysate;

(c) the geometric mean of the end-point concentration of solutions of series B is not more than 2λ or not less than 0.5λ.

If the result obtained is outside the specified limit, the test preparation under examination is acting as an inhibitor or enhancer. The interfering factors may be eliminated by further dilution (not greater than MVD), filtration, neutralisation, inactivation or by removal of the interfering substances. The use of a more sensitive lysate permits the use of greater dilution of the preparation under examination.

Ultrafiltration with suitable consumables or affinity chromatography may be used, if necessary, when the interfering factor passes through a filter with a nominal separation limit corresponding to a molecular weight of 10000 to 20000. Such filters should be checked for the presence of any factors causing false positive results. The material retained on the filter, which contains the endotoxins, is rinsed with *water BET or tris-chloride buffer pH 7.4 BET*. The endotoxins are recovered in the *water BET* or the buffer. The endotoxin concentration in the test volume and the final volume are determined for each preparation under examination.

Establish that the chosen treatment effectively eliminates interference without removing endotoxins by repeating the test for interfering factors using the preparation under examination to which the CSE has been added and which has been submitted to the chosen treatment.

Method A. Gel-Clot Limit Test Method

Preparation of test solutions. Unless otherwise prescribed, prepare the solutions and dilutions with *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1M hydrochloric acid BET*, *0.1M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Prepare the sample solution at any dilution at or below MVD. Use *water BET* as negative control (NC) and two positive controls. One of the positive controls consists of the CSE at a concentration of 2λ and the other consists of the test solution spiked with CSE to give a concentration of 2λ (PPC).

Method. Carry out the procedure on the test solutions in duplicate as described under Test for interfering factors.

Interpretation of results. The product under examination complies with the bacterial endotoxin test if the positive product control is positive and the negative control as well as the test solutions are negative. The test is not valid if the positive product control is negative or if the negative control is positive.

The product under examination meets the requirements of the test if the endotoxin content is less than the endotoxin limit stated in the individual monograph.

Retests. If a positive result is found for one of the test solution duplicates and a negative result for the other, the test may be repeated using the same validated method as described above. The results of the retest should be interpreted as for the initial test.

Method B. Semi-Quantitative Gel-Clot Method

Preparation of test solutions. Prepare test solutions at concentrations of MVD, 0.5 MVD, 0.25 MVD or any other appropriate dilutions relative to the dilution at which the test for interfering factors was completed. Additionally, prepare a similar series of test solutions spiked with 2λ of CSE each (PPC).

Method. Carry out the procedure on the test solutions in duplicate as described under Test for interfering factors.

Calculation and interpretation of results. To calculate the endotoxin concentration in the product, determine for the series of test solutions the lowest concentration or the highest dilution giving a positive (+) reaction. Multiply this dilution factor with λ to obtain the endotoxin concentration of the product.

For instance, if MVD is equal to 8 and the positive reaction was obtained at 0.25 MVD and λ was equal to 0.125 EU per ml, the endotoxin concentration in the test solution will be

$$8 \times 0.25 \times 0.125 = 0.25 \text{ EU per ml.}$$

If none of the dilutions of the series gives a positive reaction, the endotoxin concentration will be less than the value obtained by multiplying the lowest dilution factor with λ. If all the dilutions of the series give a positive reaction, the endotoxin concentration will be more than the value obtained by multiplying the highest dilution factor with λ.

Calculate the endotoxin content of the product under examination from the endotoxin concentration. The product under examination meets the requirements of the test if the endotoxin content is less than the endotoxin limit stated in the individual monograph.

Quantitative Methods

The quantitative methods include

— Kinetic Turbidimetric Method (Method C),

— Kinetic Chromogenic Method (Method D) and

— End-Point Chromogenic Method (Method E).

These methods make use of an appropriate regression analysis of the log response with the log endotoxin concentration.

To ascertain the precision or validity of the turbidimetric and chromogenic methods, preparatory tests are conducted to see that the criteria for the standard curve are valid and that the test solution does not interfere with (inhibit or enhance) the reaction.

Method C. Kinetic Turbidimetric Method

The kinetic turbidimetric method is a photometric assay measuring the increase in turbidity caused by the reaction of the endotoxin with the lysate. The kinetic turbidimetric assay is a method measuring either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture or the rate of turbidity development.

Method D. Kinetic Chromogenic Method

The kinetic chromogenic method is a photometric assay measuring the colour developed by the chromophore released from a chromogenic substrate by the reaction of the endotoxin with the lysate. The kinetic chromogenic assay is a method measuring either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture or the rate of colour development.

Test for interfering factors. For validation of the test results, it must be demonstrated that the test preparation does not inhibit or enhance the test or otherwise interfere with the test. The validation must be repeated if the lysate vendor or the method of manufacture or formulation of the sample is changed. The initial dilution may be prepared using the following expression:

$$\text{Initial dilution} = \frac{\text{Endotoxin limit of the test solution}}{C^*}$$

*C is the lowest CSE concentration of the standard curve expressed in EU/ml.

Preparation of test solutions. Prepare solutions A to D as given below.

Solution A = Solution of the product under examination at the initial dilution (test solution).

Solution B = Test solution spiked with CSE at a concentration at or near the middle of the standard curve (PPC).

Solution C = Standard solutions of CSE in *water BET* covering the linear part of the standard curve.

Solution D = *Water BET* (NC).

The pH of the solutions must be in the range specified by the manufacturer of the lysate, usually between 6.0 and 8.0. Adjust the pH, if necessary, by addition of sterile *0.1 M hydrochloric acid BET*, *0.1 M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Method. Carry out the test in duplicate receptacles such as wells of a micro-titre plate. Into each chosen receptacle, add an appropriate volume of solution D (NC), standard CSE solutions in *water BET* (solution C), test solution (solution A) and solution B (PPC). Add the lysate and carry out the assay in accordance with the instructions given by the lysate manufacturer.

Calculation and interpretation of results. Calculate the endotoxin concentration of solutions A and B from the regression equation obtained with solutions of series C. Calculate the mean percentage recovery of the added endotoxin by subtracting the mean endotoxin concentration in solution A from the mean endotoxin concentration in solution B.

The test for interfering factors is valid only if

(a) the negative control (solution D) does not yield a value higher than the limit for the value required in the description of the lysate employed;

(b) the CSE solutions of series C comply with the requirements given under Preparation of the standard curve;

(c) the mean percentage recovery of added endotoxin in solution B is between 50 per cent and 200 per cent.

If the mean percentage recovery is beyond the specified range, the interfering factors must be removed by the procedure described under the Gel-Clot Method.

Preparation of the standard curve. Using CSE, prepare solutions of 3 point, 2 log curve for endotoxin concentrations to obtain a linear standard curve. Carry out the procedure using at least two replicates of each standard endotoxin solution in accordance with the instructions of the lysate manufacturer (volume ratios, incubation times, temperature, pH, etc.).

The regression line must have linearity with the coefficient of correlation, r, greater than or equal to 0.980 or ≥ 0.980 for the range of endotoxin concentrations.

Preparation of test solutions. Unless otherwise prescribed, prepare the solutions to be employed in the test using *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1 M hydrochloric acid BET*, *0.1 M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Prepare the test solution at a suitable dilution. Use not less than three CSE concentrations to prepare a linear standard

curve. Use *water BET* as negative control and one positive control. The positive control consists of the test solution spiked with CSE to give an endotoxin concentration at the middle or below the middle point of the standard curve (PPC).

Method. Carry out the procedure described under Test for interfering factors.

Interpretation of results. The assay is valid only if

(a) the standard curve is linear for the range of CSE concentrations used;

(b) the absolute value of the coefficient of correlation is greater than or equal to 0.980;

(c) the mean percentage recovery of the added endotoxin in the positive product control is between 50 per cent and 200 per cent.

The product under examination meets the requirements of the test if the mean endotoxin content of the replicates, after correction for dilution and concentration, is less than the endotoxin limit stated in the individual monograph.

Method E. End-Point Chromogenic Method

The end-point chromogenic method is a photometric assay measuring the colour developed by the chromophore released from a chromogenic substrate by the reaction of the endotoxin with the lysate. The end-point assay is a method measuring the colour intensity at the end of an incubation period after the reaction is stopped by the addition of a suitable acid.

Preparation of test solutions. Unless otherwise prescribed, prepare the solutions to be employed in the test using *water BET*. If necessary, adjust the pH of the solution under examination to 6.0 to 8.0 using sterile *0.1M hydrochloric acid BET*, *0.1M sodium hydroxide BET* or a suitable buffer prepared with *water BET*.

Prepare the test solution at a suitable dilution. Prepare a reagent blank and not less than three dilutions of CSE in *water BET* to prepare a linear standard curve. Use *water BET* as negative control and one positive control. The positive control consists of the test solution spiked with CSE to give an endotoxin concentration at the middle or below the middle point of the standard curve (PPC).

Method. Carry out the procedure described under Test for interfering factors. The chromogenic substrate and lysate are added to the solution and incubated for the recommended time. Stop the reaction and measure the absorbance at the wavelength specified by the lysate manufacturer.

Perform the linear regression analysis of the absorbance on the endotoxin concentration using standard statistical methods (method of least squares is usually suitable). Do not average the absorbance values of the replicates of each standard before performing the linear correlation regression analysis. Determine the endotoxin concentration of the test solution from the standard curve.

Interpretation of results. The assay is valid only if

(a) the standard curve is linear for the range of CSE concentrations used;

(b) the co-efficient of correlation, r, greater than or equal to 0.980 or ≥ 0.980.

(c) The mean percentage recovery of the added endotoxin in positive product control is between 50 per cent and 200 per cent.

The product under examination meets the requirements of the test if the mean endotoxin content of the replicates, after correction for dilution and concentration, is less than the endotoxin limit stated in the individual monograph.

2.2.4. Depressor Substances

Special Reagents

Heparinised saline solution. A sterile *saline solution* containing 50 Units of heparin in 1 ml.

Standard histamine solution. Dissolve a suitable quantity of *histamine dihydrochloride* or *histamine acid phosphate* in sufficient *water* or *saline solution* to produce a solution containing 0.1 mg of histamine, $C_5H_9N_3$, per ml. Make suitable dilutions with the solvent used for preparing the solution.

Test Animal

Use a healthy, adult cat, either male or non-pregnant female, weighing not less than 2 kg. Weigh the cat and anaesthetise it by intraperitoneal injection of an anaesthetic substance such as chloralose or a suitable barbiturate that allows maintenance of a uniform blood pressure. Immobilize the animal, protect it from loss of body heat and maintain it so that the rectal temperature remains within physiological limits. Introduce a tube into the trachea. Expose a carotid or other suitable artery, separate it from surrounding tissues, insert a cannula filled with heparinised saline solution and connect to a device capable of recording the blood pressure continuously. Then expose a femoral vein and insert another cannula filled with heparinised saline solution to facilitate intravenous injection of solutions of histamine and of the substance under examination.

Determine the sensitivity of the animal to histamine by injecting intravenously, at regular intervals of not less than 5 minutes, doses of standard histamine solution corresponding to 0.05, 0.1 and 0.15 µg of histamine per kg of body weight of the animal. Repeat the injection of the dose of 0.1 µg per kg at least three times. Administer the second and subsequent injections not less than 1 minute after the blood pressure has returned to a constant level. Use the animal for the test only if

the responses to the graded doses are clearly different and the responses to the repeated injections of the dose of 0.1 µg per kg are approximately the same and correspond to a decrease in pressure of not less than 20 mm of mercury.

Method

Dissolve the substance under examination in sufficient *saline solution* or other diluent prescribed in the individual monograph, to give the test solution of the concentration specified in the monograph. Follow the same time schedule established during the injection of standard histamine solution. Inject intravenously per kg of the cat's weight, 1.0 ml of standard histamine solution followed by an injection of the specified amount of the test solution and finally 1.0 ml of standard histamine solution. The second and third injections are given not less than 1 minute after the blood pressure has returned to a constant level. When a common cannula is used for both the standard histamine solutions and test solutions, each injection of the standard and the test solutions should be immediately followed by an injection of approximately 2.0 ml of *saline solution* to flush any residues from the tubing. Measure the change in blood pressure following each of the three injections. The depressor response to the test solution is not greater than one-half of the mean depressor response to the two associated doses of the standard histamine solution. If this requirement is not met, continue the series of injections similarly until it consists of five doses, of which the three doses of 1.0 ml each of standard histamine solution are alternated with two doses of the test solution. Measure the change in blood pressure following each of the additional injections. The substance passes the test if the depressor response to each dose of the test solution is not greater than the mean of the respective depressor responses to the associate doses of the standard histamine solution represen-ting 0.1 µg of histamine per kg.

If the depressor response to either dose of the test solution is greater than the mean of the depressor response to the associated doses of the standard histamine solution the test may be continued in the same animal or in another animal similarly prepared and tested for responses to the standard histamine solution. If the test continued in the same animal after the last dose of the standard histamine solution of the initial series, administer four more injections, of which two are doses of the test solution and two are doses of 1.0 ml each of the standard histamine solution alternately in sequence. If the test is continued in another animal, prepare a fresh solution of the substance under examination from an independent container or containers of the substance and inject a series of five doses comprising the standard histamine solution and test solution in accordance with the initial injection sequence. Measure the change in blood pressure following each of the additional injections. Compute the difference between each response to the dose of the test solution and the mean of the associated doses of the standard histamine solution in the entire series, initial and additions, and calculate the average of all such differences is such that in the specified dose the depressor response to the test solution is not greater than the depressor response to the dose of the standard histamine solution representing 0.1 µg of histamine per kg and if not more than one-half of the depressor responses to the test solution are greater than the mean of the respective depressor responses to the associated doses of the standard histamine solution representing 0.1 µg of histamine per kg.

2.2.5 Test for Colony Forming Units (CFU)

The number of Colony Forming Units (CFU) must be determined on the contents of at least 5 containers of the freeze-dried vaccine. If the containers are vacuum sealed, check for vacuum before use.

Special reagents

1. Dilute Sauton's Medium

a. *Sauton's fluid medium*

Ferric ammonium citrate (brown)	0.05 g
L-Asparagine	4.0 g
Citric acid	2.0 g
Magnesium sulphate ($MgSO_4,7H_2O$)	0.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5 g
Glycerin	60.0 ml
Distilled water to	1000 ml

Dissolve the solid ingredients in 50 ml of *distilled water* by warming on a water-bath. Add glycerin and sufficient *distilled water* to produce 1000.0 ml , mix well and filter. Adjust the pH of the filtrate to 7.2 ± 0.2 with *5 M sodium hydroxide*. Sterilise by heating at 121° for 30 minutes. Store the medium in a light resistant container in a cold place.

b. *Phosphate buffer solution*

Dipotassium hydrogen phosphate	1.452 g
Sodium dihydrogen phosphate	7.601 g
Sodium chloride	4.8 g
Distilled water to	1000 ml

Dissolve the solids in sufficient *distilled water* to produce 1000.0 ml. Warm on a water-bath, if necessary, and filter.

c. *Polysorbate 80 solution*

Polysorbate 80	10 ml
Phosphate buffer solution	90 ml

Mix and sterilise by heating at 121° for 20 minutes. Store in a cold place.

d. *Dilute Sauton's solution*

Sauton's fluid medium	1000 ml
Distilled water to	3000 ml

Mix well and adjust the pH to 7.2 ± 0.2. Distribute into suitable containers. Sterilise by heating at 121° for 20 minutes.

Add 5 ml of sterile *polysorbate solution* to 600 ml of *dilute Sauton's solution* immediately before use.

2. **Lowenstein – Jensen Medium**: LJ Medium

a. *Mineral salt solution*

Potassium hydrogen phosphate (K_2HPO_4)	2.4 g
Magnesium sulphate ($MgSO_4$)	0.24 g
Magnesium citrate	0.6 g
L-Asparagine	3.6 g
Glycerin	12.0 ml
Distilled water to	600 ml

Dissolve the solid ingredients in 50 ml of *distilled water* by warming on a water-bath. Add glycerin and sufficient amount of *distilled water* to produce 600 ml and mix well. Sterilise by heating at 121° for 25 minutes.

b. *Malachite green solution*

Prepare a 2 per cent w/v solution of *malachite green* in *sterile water* with aseptic precautions, allowing the dye to dissolve by incubating for 1 to 2 hours at 37°. Shake the solution before use.

c. *Lowenstein-Jensen solution*

Mineral salt solution	600 ml
Malachite green solution	20 ml
Egg fluid	1000 ml

NOTE — All utensils used to prepare the medium must be sterile. The eggs must be fresh, i.e. not more than 4 days old. About 20 to 22 eggs depending on size, will be required to provide a litre of egg fluid.

Wash the eggs thoroughly in warm water with a brush and a plain alkaline soap, rinse them in running water for 30 minutes, drain off water and allow the eggs to dry covered with paper until the following day. Alternatively, dry them at once by sprinkling them with methylated spirit and burning it off. Crack the eggs with a sterile knife, pour the contents into a sterile beaker and beat the whites and yolks together with sterile egg whisk until a uniform egg fluid mixture free from air bubbles is obtained.

To the mixed egg fluid add the *mineral salt solution* and *malachite green solution* with aseptic precautions, mix thoroughly, distribute 5 ml aliquots into suitable bottles and screw on the caps tightly. Alternatively container like tissue culture flask with screw cap of appropriate size may be used. Lay the bottles horizontally in the inspissator. A hot air oven fitted with a fan may be used for inspissation. Preheat the oven to 85° and place the shelves on which the bottles of medium have been laid horizontally. When the temperature reaches 80° adjust the thermostat to this level and continue heating for another 60 minutes to coagulate and solidify the medium.

Method

Reconstitute each of 5 containers of the freeze-dried vaccine for human use with suitable diluents and pool the contents. Prepare 2 fold dilutions of the test sample in such a way so as to get optimum count of 100, 40 and 20 colonies from an inoculum of 0.2 ml by using *dilute Sauton's medium* for preparing the dilutions. Ideally middle dilution should yield nearly 40 colonies per container. Normally, dilutions in the range of 1:20,000, 1:40,000 and 1:80,000 would be required.

Inoculate 0.2 ml of each of the dilutions on to *LJ medium* by surface inoculation. Use three bottles of *LJ medium* for the first and second dilutions each and six bottles for the third dilution. Incubate the inoculated *LJ medium* at 37° ± 1° for 4 to 5 weeks and count the number of colonies. Calculate the number of culturable particles by statistical tool as per WHO guideline or any other statistical tool which give full weightage to a dilution, yielding on an average, the optimum or lesser number of colonies, reduced weightage to a dilution yielding up to twice the optimum number of colonies and no weightage to a dilution yielding more than twice the optimum number of colonies.

The vaccine passes the test if the estimated count is within the limit as stated on the label. The difference between the lower and upper specification for the number of culturable particles should not be larger than four-fold.

NOTE — The validity of the test for colony-forming units (CFU) must be determined by carrying out the test on a preparation of known potency.

2.2.6. Haemolysins

Add 1 volume of fresh donor serum to 1 volume of a 10 per cent v/v suspension of A_1 corpuscles in *saline solution* and add 1 volume to 1 volume of a similar suspension of B corpuscles; a similar test using O corpuscles may be done as a negative control. If the serum is more than 24 hours old, add 1 volume of fresh group O serum, free of lysins, to each tube as a source of complement. Mix the contents of each tube, incubate at 37° for 1 hour and examine the supernatant liquid for haemolysis.

A serum giving a positive result in this test is further examined as follows. Dilute 1 volume of the serum with 3 volumes of *saline solution* and mix 1 volume of the diluted serum with

1 volume of fresh group O serum free of lysins and 1 volume of a 10 per cent v/v suspension, in *saline solution,* of A_1 or B corpuscles (whichever were lysed in the first test). At the same time, in two further tubes, mix 1 volume of *saline solution* with 1 volume of the fresh group O serum free of lysins. To one of these tubes, add 1 volume of a 10 per cent v/v suspension, in *saline solution,* of A_1 corpuscles and to the other 1 volume of a similar suspension of B corpuscles. Incubate the tubes at 37° for 1 hour, mix the contents of each tube and examine the supernatant liquid for haemolysis. No haemolysis should show in any of the tubes. Group O blood samples whose sera show the presence of haemolysins should be regarded as unsafe for transfusion to recipients of other groups and must be labelled accordingly.

2.2.7. Histamine

Solutions

Solution 1

Sodium chloride	160.0 g
Potassium chloride	4.0 g
Calcium chloride, anhydrous	2.0 g
Magnesium chloride, anhydrous	1.0 g
Disodium hydrogen phosphate dodecahydrate	0.10 g
Water for injections to	1000 ml

Solution 2

Solution 1	50.0 ml
Atropine sulphate	0.5 mg
Sodium bicarbonate	1.0 g
Dextrose monohydrate	0.5 g
Water for injections to	1000 ml

Solution 2 should be freshly prepared and used within 24 hours.

Method

Kill a guinea-pig weighing 250 g to 350 g that has been deprived of food for the preceding 24 hours. Remove a portion of the distal small intestine 2 cm long and empty the isolated part by rinsing carefully with solution 2 using a syringe. Attach a fine thread to each end and make a small transverse incision in the middle of the piece of intestine. Place it in an organ bath with a capacity of 10 ml to 20 ml, containing solution 2 maintained at a constant temperature of 34° to 36° and pass through the solution a current of a mixture of 95 parts of oxygen and 5 parts of carbon dioxide. Attach one of the threads near to the bottom of the organ bath. Attach the other thread to an isotonic myograph and record the contractions of the organ on a kymograph or any other suitable means of giving a permanent record. If a lever is used, its length is such that the movements of the organ are amplified about 20 times. The tension on the intestine should be about 9.8 mN (1 g) and it should be adjusted to the sensitivity of the organ. Flush out the organ bath with solution 2. Allow to stand for 10 minutes. Flush 2 or three times more with solution 2. Stimulate a series of contractions by the addition of measured volumes between 0.2 ml and 0.5 ml of a solution of *histamine dihydrochloride* having a strength that produces reproducible submaximal responses. This dose is the high dose. Flush the organ bath 3 times with solution 2 before each addition of histamine. The successive additions should be made at regular intervals allowing a complete relaxation between additions (about 2 minutes). Add equal volumes of a weaker dilution of *histamine dihydrochloride* which produces reproducible responses approximately half as great as the high dose. This dose is the low dose. Continue the regular additions of high and low doses of histamine solution as indicated above, and alternate each addition with an equal volume of a dilution of the solution under examination, adjusting the dilution so that the contraction of the intestine, if any, is smaller than that due to the high dose of histamine. Determine whether the contraction, if any, is reproducible and that the responses to the high and low doses of histamine are unchanged. Calculate the activity of the substance under examination in terms of its equivalent in micrograms of histamine base from the dilution determined as above.

The quantity so determined does not exceed the quantity prescribed in the individual monograph.

If the solution under examination does not produce a contraction, prepare a fresh solution adding a quantity of histamine corresponding to the maximum tolerated in the monograph and note whether the contractions produced by the preparation with the added histamine correspond to the amount of histamine added. If this is not the case, or if the contractions caused by the substance under examination are not reproducible or if subsequent responses to high and low doses of histamine are diminished, the results of the test are invalid and the test for depressor substances (2.2.4) must be done.

2.2.8. Pyrogens

The test involves measurement of the rise in body temperature of rabbits following the intravenous injection of a sterile solution of the substance under examination. It is designed for products that can be tolerated by the test rabbit in a dose not exceeding 10 ml per kg injected intravenously within a period of not more than 10 minutes.

Test Animals

Use healthy, adult rabbits of either sex, preferably of the same variety, weighing not less than 1.5 kg, fed on a complete and balanced diet and not showing loss of body weight during the week preceding the test. House the animals individually in an area of uniform temperature (± 2°), preferably with uniform humidity, and free from disturbances likely to excite them.

Do not use animals for pyrogen tests more frequently than once every 48 hours. After a pyrogen test in the course of which a rabbit's temperature has risen by 0.6° or more, or after a rabbit has been given a test substance that was adjudged pyrogenic, at least 2 weeks must be allowed to elapse before the animal is used again.

Materials

All glasswares, syringes and needles must be thoroughly washed with *water for injections* and heated in a hot air oven at 250° for 30 minutes or at 200° for 1 hour. Treat all diluents and solutions for washing and rinsing of devices in a manner that will assure that they are sterile and pyrogen-free.

The retaining boxes for rabbits in which the temperature is being measured by electrical device should be made in such a way that the animals are retained only by loosely-fitting neck-stocks and the rest of the body remains relatively free so that the rabbits may sit in a normal position. The animals must be put in the boxes 1 hour before the test and remain in them throughout the test. Ensure that the room temperature where the test is carried out is within 3° of that of the rabbits living quarters or in which the rabbits have been kept for 18 hours before the test. Withhold food from the animals overnight and until the test is completed; withhold water during the test.

Recording of Temperature

Use an accurate temperature-sensing device such as a clinical thermometer or thermistor or other suitable probes that have been calibrated to assure an accuracy of 0.1° and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the thermometer or temperature-sensing probe into the rectum of the test rabbit to a depth of about 5 cm. The depth of insertion is constant for any one rabbit in any one test. If an electrical device is used, it should be inserted in the rectum of the rabbit 90 minutes before the injection of the solution being examined and left in position throughout the test. After a period of time not less than that previously determined as sufficient, record the rabbit's body temperature.

Preliminary Test (Sham Test)

If animals are used for the first time in a pyrogen test or have not been used during the 2 previous weeks, condition them 1 to 3 days before testing the substance under examination by injecting intravenously into them 10 ml per kg of body weight of a pyrogen-free *saline solution* warmed to about 38.5°.

Record the temperatures of the animals, beginning at least 90 minutes before injection and continuing for 3 hours after injection of the solution being examined. Any animal showing a temperature variation of 0.6° or more must not be used in the main test.

Main Test

Carry out the test using a group of three rabbits.

Preparation of the sample. Dissolve the substance under examination in, or dilute with, pyrogen-free *saline solution* or other solution prescribed in the monograph. Warm the liquid under examination to approximately 38.5° before injection.

Procedure. Record the temperature of each animal at intervals of not more than 30 minutes, beginning at least 90 minutes before the injection of the solution under examination and continuing for 3 hours after the injection. Not more than 40 minutes immediately preceding the injection of the test dose, record the "initial temperature" of each rabbit, which is the mean of two temperatures recorded for that rabbit at an interval of 30 minutes in the 40-minute period. Rabbits showing a temperature variation greater than 0.2° between two successive readings in the determination of "initial temperature" should not be used for the test. In any one group of test animals, use only those animals whose "initial temperatures" do not vary by more than 1° from each other, and do not use any rabbit having a temperature higher than 39.8° and lower than 38°.

Inject the solution under examination slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 minutes, unless otherwise prescribed in the monograph. The amount of sample to be injected varies according to the preparation under examination and is prescribed in the individual monograph. The volume of injection is not less than 0.5 ml per kg and not more than 10 ml per kg of body weight. Record the temperature of each animal at half-hourly intervals for 3 hours after the injection. The difference between the "initial temperature" and the "maximum temperature" which is the highest temperature recorded for a rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Interpretation of results. If the sum of the responses of the group of three rabbits does not exceed 1.4° and if the response of any individual rabbit is less than 0.6°, the preparation under examination passes the test. If the response of any rabbit is 0.6° or more, or if the sum of the response of the three rabbits exceeds 1.4°, continue the test using five other rabbits. If not more than three of the eight rabbits show individual responses of 0.6° or more, and if the sum of responses of the group of eight rabbits does not exceed 3.7°, the preparation under examination passes the test.

2.2.9. Microbial Contamination in Nonsterile Products

The following tests are designed for the estimation of number of viable aerobic microorganisms present, for detecting the presence of designated microbial species and acceptance criteria in pharmaceutical substances, nonsterile dosage forms, herbs, processed herbs and herbal products.

1. Total aerobic viable count

Introduction

The tests described here after will allow enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

These tests are not applicable to product containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeial method has been demonstrated.

These tests should be carried out under conditions designed to avoid accidental contamination during the test. The precautions taken to avoid contamination must be such that they do not adversely affect any microorganisms that should be revealed in the test.

Before carrying out these tests for enumeration of the aerobic microorganisms in the test specimen, it should be confirmed that:

i) The media used in the tests, promote the growth of the respective microorganisms for which they are used.

ii) The test specimens to which the tests are applied do not themselves inhibit the microorganisms and the appropriateness of the methods used for enumeration of microorganism in presence of the product is determined.

Preparation of inoculum

Microorganisms used. *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404.

In order to prevent any phenotypic changes in the strains used, the organisms used in the test should not be more than 5 passages from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium.

Grow each of the bacterial test strains separately in Casein soyabean digest broth (Medium 1) and incubate at 30° to 35° for 18 to 24 hours. Grow *Candida albicans* separately in Sabouraud dextrose broth (Medium 3) or Sabouraud dextrose agar with antibiotic (Medium 4) and incubate at 20° to 25° for 48 hours. Grow *Aspergillus brasiliensis* on Sabouraud dextrose agar with antibiotic at 20° to 25° for 5 to 7 days or until good sporulation. After incubation, prepare test suspension of each organism separately in *Buffered sodium chloride-peptone solution pH 7.0*. To suspend spores of *Aspergillus brasiliensis*, 0.05 per cent *polysorbate 80* may be added to the buffered solution. Use test suspension of these organisms within 2 to 4 hours. The suspension may be stored at 2° to 8° for a validated period of time.

Growth promotion by media

Test each batch of the medium prepared either from the dehydrated medium or from the ingredients described for growth promotion. Use the already approved medium prepared from dehydrated medium or from the ingredients as a positive control.

Negative control. To verify the test conditions, use the diluent, sterile *buffered sodium chloride-peptone solution pH 7.0* as a negative control in place of the test organisms (inoculum). There should not be any growth of microorganisms in this control. If a negative control fails (microorganisms grow in the control), its cause should be investigated.

Inoculation

Inoculate 10 ml of Casein soyabean digest broth (Medium 1) with not more than 100 CFU of each of the three above mentioned species of bacteria using separate tube for each and incubate at 30° to 35° for 18 to 24 hours. Similarly inoculate plates of Casein soyabean digest agar (Medium 2) with not more than 100 CFU of each of the three above mentioned strains of organisms using separate plate of medium for each and incubate at 30° to 35° for 18 to 24 hours. Inoculate plates of Sabouraud dextrose agar with antibiotic (Medium 4) using not more than 100 CFU of *Candida albicans* and *Aspergillus brasiliensis* and incubate at 20° to 25° for 5 days.

For comparison purpose, similarly inoculate and incubate the previously approved liquid and solid medium (as a positive control) along with the media under test.

Growth

Liquid media under test should be considered suitable if growth obtained is comparable to that obtained on the same medium, previously tested and approved.

Growth obtained on a solid medium must not differ from the calculated CFU of the standardized inoculum by a factor greater than 2 or should be comparable to that obtained on the same medium previously tested and approved.

Appropriateness of enumeration methods in presence of product

Pre-treatment of the sample

Use suitable alternative method if following methods are not applicable.

Water soluble product. Dissolve 10 g or dilute 10 ml of the preparation under examination, unless otherwise specified, in *buffered sodium chloride-peptone solution pH 7.0* or any other suitable medium shown to have no antimicrobial activity under the conditions of the test and adjust the volume to 100 ml with the same. If necessary, adjust the pH to about 7.0. If required, further dilutions are prepared with the same diluent.

Products insoluble in water (non-fatty). Suspend 10 g or 10 ml of the preparation under examination, unless otherwise specified, in *buffered sodium chloride-peptone solution pH 7.0* or any other suitable medium shown to have no antimicrobial activity under the conditions of the test and adjust the volume to 100 ml with the same. If necessary, divide the preparation under examination and homogenise mechanically. A suitable surface active agent such as 0.1 per cent w/v solution of *polysorbate 80* may be added to assist the suspension of poorly wettable substances. If necessary, adjust the pH of the suspension to about 7. If required, further dilutions are prepared with the same diluent.

Fatty products. Homogenize 10 g or 10 ml of the preparation under examination in *isopropyl myristate* or unless otherwise specified, with 5 g of sterile *polysorbate 20* or *polysorbate 80*. If necessary, heat to not more than 40°. Mix carefully while maintaining the temperature in waterbath. Add 85 ml of *buffered sodium chloride-peptone solution* pH 7.0 or any other suitable medium which does not have any antimicrobial activity under the conditions of the test, heated to not more than 40°, if necessary. Maintain this temperature for the shortest time necessary for formation of an emulsion and in any case for not more than 30 minutes. Adjust the volume to 100 ml with the same medium. If necessary, adjust the pH to about 7.0. Further dilutions may be prepared using the same diluent containing a suitable concentration of sterile *polysorbate 80*.

Fluids or solids in aerosol form. In sterile conditions, transfer the product into a membrane filter apparatus or to a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal patches. Remove the protective cover sheets 'release liners' of transdermal patches using sterile forceps and place them adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze and transfer them to a suitable volume of *buffered sodium chloride-peptone solution* pH 7.0 containing inactivator such as *polysorbate 80* or *lecithin*. Shake the preparation vigorously for at least 30 minutes.

Inoculation

Add to the sample prepared as mentioned above and to a control (only diluent without prepared sample) a sufficient volume of microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the inoculum should not exceed 1 per cent of the volume of the diluted product.

Carry out inoculation using each of the microorganisms separately, like this, with and without product under examination.

Use the lowest possible dilution factor of the prepared sample to demonstrate acceptable microbial recovery from the product. If the product has antimicrobial property, then the inoculum may be added after inactivation, dilution or filtration.

Inactivation of antimicrobial activity

If the test specimen is known to contain any of the below mentioned antimicrobial substances then use the corresponding inactivating agent to neutralize the antimicrobial activity (Table 1). The inactivating agent may be added to the chosen diluent or the medium preferably before sterilization. Before using the inactivating agent, verify its efficiency against the antimicrobial substance and also confirm that the inactivating agent does not have toxic effect on the organisms used in the test by carrying out a blank with the inactivator and without product. The inhibitory effect can be removed by increasing the volume of the diluent or culture media. If the inhibitory substances are water-soluble then membrane filtration method may be used. If inspite of incorporation of suitable inactivating agent and substantial increase in the volume of the diluent, it is still not possible to recover the viable culture from the product then it can be assumed that the failure to isolate the inoculated organisms may be due to microbicidal property of the product and the product is not likely to be contaminated with the microorganisms inoculated but can contain other organisms. Then carry out the tests with highest dilution factor compatible with microbial growth and the specific acceptance criteria.

Table 1–Antimicrobial substances with corresponding inactivating agents

Antimicrobial substances	Inactivator	Concentration
Phenolics, Parahydroxy benzoate (Parabens)	Polysorbate 80	30 g per litre
Iodine, Quaternary ammonium compound (QACs)	Lecithin Sodium lauryl sulphate	3 g per litre 4 g per litre
Alcohol, Aldehydes, Sorbates	Dilution	–
Mercurial halogens	Sodium thiosulphate	5 g per litre

Enumeration of aerobic organisms in presence of product

For each of the microorganisms listed, separate tests are performed. Only microorganisms of the added test strains are counted.

Membrane Filtration

Use membrane filters 50 mm in diameter and having a nominal pore size of not greater than 0.45 µm the effectiveness of which in retaining bacteria has been established for the type of preparation under examination. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be examined. Cellulose nitrate filters may be used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters for strongly alcoholic solutions. For each of the microorganisms listed, one membrane filter is used.

Transfer a suitable quantity of the sample prepared as described under *pre-treatment of the sample, inoculum, inoculation and inactivation of the antimicrobial activity* to the membrane filter. The prepared sample should contain 1 g of the product. However, if large numbers of microorganisms are expected in the sample, then lesser quantity may be taken.

Wash each membrane by filtering through it appropriate quantity of a suitable liquid such as *buffered sodium chloride-peptone solution pH 7.0*. For determination of total aerobic count (TAC), transfer the membrane filter to the surface of Casein soyabean digest agar (Medium 2) and incubate the plates at 30° to 35° for 3 days. For total fungal count (TFC), transfer the membrane to the surface of Sabouraud dextrose agar with antibiotic (Medium 4) and incubate the plates at 20° to 25° for 5 days. Count the number of colonies that are formed and calculate the number of microorganisms per g or per ml of the product under examination.

Plate count methods

Pour-plate method. To a 9 cm diameter Petri dish add 1 ml of the sample prepared as described in *pre-treatment of the sample, inoculum, inactivating agents if necessary* and 15 ml of Casein soyabean digest agar (Medium 2) or Sabouraud dextrose agar with antibiotic (Medium 4), at not more than 45°. Use at least two Petri dishes for each of the test organisms. Incubate the plates of Casein soyabean digest agar (Medium 2) at 30° to 35° for 3 days. Similarly incubate the plates of Sabouraud dextrose agar with antibiotic (Medium 4) at 20° to 25° for 5 days. Calculate the mean count on each medium and from that calculate the number of CFU.

Surface-spread method. Using Petri dishes of 9 cm diameter add 15 ml of Casein soyabean digest agar (Medium 2) for cultivation of aerobic microorganisms or Sabouraud dextrose agar with antibiotic (Medium 4) for cultivation of fungi, at temperature about 45°, to each Petri dish and allow to solidify. Dry the plates, in an LAF bench or in an incubator. Spread a measured volume of not less than 0.1 ml of the sample prepared as described earlier, over the surface of the medium. Use atleast two Petri dishes for each medium and each strain of test organism. For incubation and calculation of the number of colony forming units proceed as described in the pour-plate method.

Most probable number method

This method (originally known as multiple-tube or serial dilution method) is to be followed when no other method is available. The procedure and accuracy of the method is less than that of the membrane filtration method or the plate count methods. Hence this method is carried out when no other method is available. If use of this method is justified, then proceed as follows

Prepare a series of at least three subsequent ten fold dilutions of the product as described in *pre-treatment of the sample, inoculum, inactivating agents if necessary*. From each level of dilution three aliquots of 1 g or 1 ml are used to inoculate three tubes with 9.0 ml of sterile Casein soyabean digest broth (Medium 1) If necessary, *polysorbate 80* or an inactivator of antimicrobial agents (Table 1) may be added to the medium. Thus, if three levels of dilution are prepared, 9 tubes are inoculated. Incubate all the tubes for three days at 30° to 35°. Record for each level of dilution the number of tubes showing microbial growth. If detection of growth is difficult or uncertain owing to the nature of the product under examination, sub-culture in the same broth, or on a suitable agar medium such as Casein soyabean digest agar (Medium 2) for 18 to 24 hours at 30° to 35°. Determine the most probable number of bacteria per g or ml of the product from Table 2.

Result

The number of microorganisms recovered from the prepared sample, inoculated and processed as mentioned above is compared with that of the number of microorganisms recovered from the control (without test specimen) preparation.

i) If growth in presence of product is inhibited by a factor >2, it indicates that the product has antimicrobial property hence process the sample as described under inactivation of antimicrobial activity and carry out the test as mentioned under *Enumeration of aerobic organisms in presence of product* and then interpret the result as mentioned under *Interpretation*.

ii) If growth is not inhibited, interpret the results as under.

Interpretation

Membrane filtration or a plate-count method is considered suitable, if mean count of any of the test organisms do not differ by a factor >2 from the mean count of the control (in absence of the product). When verifying the validity of MPN method, calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If this criteria cannot be met for any one or more of the organisms tested with any of the described methods, the

Table 2– Most-probable-number (MPN) values of microorganisms, 3 tubes at each level of dilution

Observed combinations of Numbers of tubes showing growth in each set			MPN per g or per ml of the product	95 per cent Confidence limits
Number of g or ml of product per tube				
0.1	0.01	0.001		
0	0	0	<3	0–9.4
0	0	1	3	0.1–9.5
0	1	0	3	0.1–10
0	1	1	6.1	1.2–17
0	2	0	6.2	1.2–17
0	3	0	9.4	3.5–35
1	0	0	3.6	0.2–17
1	0	1	7.2	1.2–17
1	0	2	11	4–35
1	1	0	7.4	1.3–20
1	1	1	11	4–35
1	2	0	11	4–35
1	2	1	15	5–38
1	3	0	16	5–38
2	0	0	9.2	1.5–35
2	0	1	14	4–35
2	0	2	20	5–38
2	1	0	15	4–38
2	1	1	20	5–38
2	1	2	27	9–94
2	2	0	21	5–40
2	2	1	28	9–94
2	2	2	35	9–94
2	3	0	29	9–94
2	3	1	36	9–94
3	0	0	23	5–94
3	0	1	38	9–104
3	0	2	64	16–181
3	1	0	43	9–181
3	1	1	75	17–199
3	1	2	120	30–360
3	1	3	160	30–380
3	2	0	93	18–360
3	2	1	150	30–380
3	2	2	210	30–400
3	2	3	290	90–990
3	3	0	240	40–990
3	3	1	460	90–1980
3	3	2	1100	200–4000
3	3	3	>1100	–

method and the test conditions that come closest to the criteria are used to test the product.

Testing of Products

Sampling

Sampling of the product must follow a well-defined sampling plan that takes into account the batch size, the characteristics of the product, the health hazards associated with highly contaminated products and the expected level of contamination. Unless otherwise stated, use 10 ml or 10 g specimens for testing. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g. tablet, capsule, injection) is less than or equal to 1 mg, or the amount per g or ml (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 ml of the product.

Select the samples at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of sufficient number of containers to obtain the sample.

For materials used as active ingredients where the batch size is less than 1 kg or 1 litre, the amount tested shall be 1 per cent of the batch-size.

For the products where the total number of units in a batch is less than 200, the sample size may be reduced to two units or one unit, if the size is less than 100.

Enumeration of aerobic microorganisms present in the product

Membrane Filtration

Prepare the sample using the method that has been shown to be appropriate as described in appropriateness of enumeration methods in presence of product. Transfer appropriate amount to each of the two membrane filters and filter immediately. Wash each filter following the procedure found to be suitable. For determination of total aerobic microbial count transfer one of the membrane filters to Casein soyabean digest agar (Medium 2). Incubate the plate at 30° to 35° for 3 to 5 days. For total yeast and mould count transfer the other membrane to the surface of Sabouraud dextrose agar with antibiotic (Medium 4) and incubate at 20° to 25° for 5 to 7 days. Calculate the number of CFU per g or per ml of the product.

Plate count methods

Pour-plate method. Prepare the sample using a method that has shown to be suitable as described in Appropriateness of the enumeration methods in presence of product. Stir the prepared sample in the tubes/flasks vigorously on a vortex mixer for few minutes. Each dilution of the product sample should be stirred like this. Use atleast two plates of Casein soyabean digest agar for each dilution. Incubate the plates at 30° to 35° for 3 to 5 days. Similarly for total fungal count use atleast two plates of Sabouraud dextrose agar with antibiotic for each dilution and incubate at 20° to 25° for 5 to 7 days. Then select the plates corresponding to a given dilution and showing the highest number of colonies but less than 250 for aerobic microorganisms and 25 colonies for fungi. Calculate the mean count and number of CFU per g or per ml of the product.

Surface-spread method. Prepare the sample using a method that has been shown to be suitable as described in Appropriateness of the enumeration methods in presence of product and further process it as mentioned in the pour-plate method above. Spread 0.1 ml of the dilution. Use atleast two plates for each medium and each of the dilutions. Incubate, select and calculate the number of CFU as mentioned in *pour plate method.*

Most probable number method

Prepare and dilute the sample using a method that has been shown to be appropriate as described in the *Growth promotion test* and *Appropriateness of the enumeration method.* Incubate all tubes at 30° to 35° for atleast 3 days. For each level of dilution note down the number of tubes showing microbial growth. Subculture if required as described before. Determine the most probable number of microorganisms per g or per ml of the product as per Table 2.

Interpretation of the results

The total aerobic viable count (TAC) is considered to be equal to the number of CFU found on Casein soyabean digest agar. If colonies of fungi are detected on this medium, they are counted as part of TAC. The total fungal count (TFC) is considered to be equal to the number of CFU found using Sabouraud dextrose agar with antibiotic.

Acceptance criteria for microbiological quality should be interpreted as follows:

10^1 CFU : maximum acceptable count 20

10^2 CFU : maximum acceptable count 200

10^3 CFU : maximum acceptable count 2000, and so forth....

2. Tests for specified microorganisms

The tests described below will allow determination of the absence of, or limited occurrence of specified microorganisms that may be detected under the conditions described.

These tests are carried out to find out whether a substance or a preparation complies with an established specification for microbiological quality.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the pharmacopoeial method has been demonstrated. Maintenance, Identification, Preservation and Disposal of Microorganisms are mentioned in General Chapter (2.2.20).

Following procedures are carried out as described under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9).

i) The preparation of sample.
ii) Inactivation of antimicrobial property, if present, in the product.
iii) Confirmation that the surface active substances if used, are not toxic for the test-organisms and are compatible with the inactivating agent.

Growth promotive and selective properties of the media and validity of the test

Test each batch of the medium prepared either from the dehydrated medium or from the ingredients described for growth promotion. Use the already tested and approved medium prepared from dehydrated medium or from the ingredients as a positive control. Verify the properties of relevant media as described in Table 3:

Preparation of the inoculum

Microorganisms used. *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739 , *Salmonella enterica spp. enterica serotype typhimurium* ATCC 14028 or *Salmonella enterica spp. enterica serotype abony* NCTC 6017, *Shigella boydii* ATCC 8700 or NCTC 12985, *Candida albicans* ATCC 10231, *Clostridium sporogenes* ATCC 11437 or ATCC 19404.

In order to prevent any phenotypic changes in the strains used, the organisms used in the test should not be more than 5 passages made from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium.

Grow each of the aerobic bacterial strains separately in Casein soyabean digest broth (Medium 1) and incubate them at 30° to 35° for 18 to 24 hours. Grow *Candida albicans* in Sabouraud dextrose broth (Medium 3) and incubate at 20° to 25° for at least 48 hours. After incubation, re-suspend the growth of each of the organisms separately in *buffered sodium chloride-peptone solution pH 7.0*. Use suspension of these organisms within 2 to 4 hours. The suspension may be stored at 2° to 8°. for a validated period of time.

Grow *Clostridium sporogenes* ATCC 11437 under anaerobic conditions in Reinforced medium for *Clostridia* (Medium 15) at 30° to 35° for 24 to 48 hours. Instead of vegetative cells a spore suspension may be used for inoculation.

Negative control. To test the sterility of the medium and the diluent, use the diluent, *buffered sodium chloride-peptone solution pH 7.0* (without organisms) as a negative control. There should not be any growth of microorganisms in this control. If a negative control fails (microorganisms grow in the control), its cause should be investigated.

Tests for growth promotion by liquid media. Inoculate 10 ml of the appropriate medium with not more than 100 CFU of the appropriate test microorganisms. Incubate at the specified temperature for the time specified in the test. Clearly visible growth of the microorganism comparable to that obtained with previously approved batch of medium occurs.

Tests for growth promotion by solid media. Using the surface spread method as described under *Total viable aerobic count* in *Microbial contamination in nonsterile products* (2.2.9), inoculate each plate with not more than 100 CFU of appropriate microorganism. Incubate at the specified temperature for the time specified in the test. Growth obtained on the medium should be comparable to that on the same medium previously approved.

Tests for growth inhibition in liquid or solid media. Inoculate the appropriate medium with not less than 100 CFU of the appropriate microorganism. Incubate at the specified temperature for the time specified in the test. There should not be any growth of the microorganism on this medium.

Tests for indicative properties. Using the surface spread method as described under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9). Inoculate each plate with not more than 100 CFU of appropriate microorganism. Incubate at the specified temperature for the time specified in the test. Colony morphology and indication reactions should be similar to that obtained with the previously approved batch of medium.

Validity of the test method. For each new product to be tested prepare a sample as described below in the pertinent paragraph under Testing of Products. At the time of mixing add each test organism in the prescribed growth medium. Inoculate not more than 100 CFU of the test organisms individually. Carry out the test as described under Testing of Products. The specified microorganism must be detected with the colony morphology and indication reaction as described.

If the product has antimicrobial activity then it should be inactivated as mentioned in inactivation of antimicrobial activity under appropriateness of enumeration method in presence of product. If the antimicrobial activity of a given product cannot be inactivated then it is assumed that the inhibited microorganism will not be present in the product.

Testing of Products

Bile-Tolerant Gram-Negative Bacteria

Preparation of Sample and Incubation. Using Casein soyabean digest broth (Medium 1) as a diluent, make 1 in 10

Table 3–Growth promoting, inhibitory and indicative properties of media

Test/Medium	Property	Test Strains
Test for bile tolerant Gram-negative bacteria		
Enterobacteria Enrichment Broth Mossel	Growth promoting	*E. coli, P. aeruginosa*
	Inhibitory	*S. aureus*
Violet Red Bile Glucose Agar	Growth promoting + Indicative	*E. coli*
		P. aeruginosa
Test for *Escherichia coli*		
MacConkey Broth	Growth promoting	*E. coli*
	Inhibitory	*S. aureus*
MacConkey Agar	Growth promoting + Indicative	*E. coli*
Test for *Salmonella*		
Rappaport Vassiliadis Salmonella Enrichment Broth	Growth promoting	*Salmonella enterica spp. enterica serotype typhimurium or Salmonella enterica spp. enterica serotype abony*
	Inhibitory	*S. aureus*
Wilson and Blair's BBS Agar*	Growth promoting + Indicative	*Salmonella enterica spp. enterica serotype typhimurium or Salmonella enterica spp. enterica serotype abony*
	Inhibitory	*E. coli, Shigella boydii*
Test for *Shigella*		
GN Medium	Growth promoting	*Shigella boydii*
	Inhibitory	*S. aureus*
Xylose Lysine Deoxycholate Agar	Growth promoting + Indicative	*Shigella boydii*
	Indicative	*E. coli*
Test for *Pseudomonas aeruginosa*		
Cetrimide Agar	Growth promoting	*P. aeruginosa*
	Inhibitory	*E. coli*
Test for *Staphylococus aureus*		
Mannitol Salt Agar	Growth promoting + Indicative	*S. aureus*
	Inhibitory	*E. coli*
Test for *Clostridia*		
Reinforced Medium for *Clostridia*	Growth promoting	*C. sporogenes*
Columbia Agar	Growth promoting	*C. sporogenes*
Test for *Candida albicans*		
Sabouraud Dextrose Broth or Agar	Growth promoting	*C. albicans*

*Xylose Lysine Deoxycholate Agar may be used.

dilution of more than 1 g of the product to be examined as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9). Mix well and keep at 20° to 25° for about 2 to 5 hours to resuscitate the organisms.

Test for detection of organisms. From above prepared sample, take a volume corresponding to 1 g of the product and inoculate in Enterobacteria Enrichment Broth – Mossel (Medium 5). Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of Violet red bile glucose agar (Medium 6). Incubate at 30° to 35° for 18 to 24 hours.

The product passes the test if there is no growth of colonies of gram negative bacteria.

Quantitative evaluation. From the above mentioned prepared sample take a volume corresponding to 0.1 g, 0.01 g and 0.001 g (or 0.1 ml, 0.01 ml, and 0.001 ml) of the product in suitable quantity of Enterobacteria enrichment Broth–Mossel (Medium 5). Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of Violet red bile glucose agar with dextrose (Medium 6). Incubate at 30° to 35° for 18 to 24 hours. Growth of well developed reddish colonies of Gram negative bacteria is considered positive. Note the smallest quantity of the product that gives the positive result and the largest quantity that gives the negative result. Determine from Table 4 the most probable number of bacteria.

Table 4 – Quantitative determination of most probable number of bacteria

Results for each quantity of product			Probable number of bacteria per g or ml of product
0.1 g or 0.1 ml	0.01 g or 0.01 ml	0.001 g or 0.001 ml	
+	+	+	More than 10^3
+	+	–	Less than 10^3 and more than 10^2
+	–		Less than 10^2 and more than 10
–	–	–	Less than 10

Escherichia coli

Using Casein soyabean digest broth (Medium 1) as a diluent make 1 in 10 dilution of more than 1 g of the product as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9) and use 10 ml or the quantity corresponding to 1 g or 1 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method)* of Casein soyabean digest broth, incubate at 30° to 35° for 18 to 24 hours.

After incubation shake the broth and transfer 1 ml to 100 ml of MacConkey broth (Medium 7). Incubate at 42° to 44° for 24 to 48 hours. Subculture on a plate of MacConkey agar (Medium 8) and incubate at 30° to 35° for 18 to 72 hours. Growth of pink, non-mucoid colonies indicates the possible presence of *Escherichia coli*. This should be confirmed by identification test.

If there is no growth of such type of colonies, or the identification tests are negative it indicates absence of *E. coli* and the product passes the test.

Salmonella

Prepare a sample from the product as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9) and use the quantity corresponding to 10 g or 10 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method.)* of Casein soyabean digest broth incubate at 30° to 35° for 18 to 24 hours.

After incubation shake the broth and transfer 0.1 ml to 10 ml of Rappaport Vassiliadis Salmonella enrichment broth (Medium 9) and incubate at 30° to 35° for 24 to 48 hours. Subculture on a plate of Wilson and Blair's BBS Agar (Medium 10) and incubate at 30° to 35° for 24 to 48 hours. Green colonies with black center develop and in 48 hours the colonies become uniformly black. Colonies surrounded by a dark zone and metallic sheen indicates possibility of presence of *Salmonella*. If sub cultured on plates of Xylose lysine deoxycholate agar and incubate at 30° to 35° for 24 to 48 hours. Well devolped, red colonies with or without black centers indicates possibility of *Salmonella*. This should be confirmed by identification tests.

If there is no growth of such type of colonies, or identification tests are negative it indicates absence of *Salmonella* and the product passes the test.

Shigella

Prepare a sample from the product to be examined as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9) and use the quantity corresponding to 10 g or 10 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method.)* of Casein soyabean digest broth incubate at 30° to 35° for 18 to 24 hours.

After incubation shake the growth and transfer 1 ml to 100 ml of GN Broth (Medium 11) and incubate at 30° to 35° for 24 to 48 hours. Subculture on a plate of Xylose lysine deoxycholate medium (Medium 12). Incubate at 30° to 35° for 24 to 48 hours. A red colored translucent colony without black centre indicates possibility of presence of *Shigella*. This should be confirmed by identification tests.

If there is no growth of such colonies or if identification tests are negative, it indicates absence of *Shigella* and the product passes the test.

Pseudomonas aeruginosa

Using Casein soyabean digest broth as a diluent make 1 in 10 dilution of more than 1 g of the product as mentioned in *Total aerobic viable count* under *Microbial contamination in nonsterile products* (2.2.9) and use 10 ml or the quantity corresponding to 1 g or 1 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method*) of Casein soyabean digest broth incubate at 30° to 35° for 18 to 24 hours. Subculture on a plate of Cetrimide agar (Medium 13) and incubate at 30° to 35° for 18 to 72 hours. A greenish color colony indicates the possibility of presence of *Pseudomonas aeruginosa*. This should be confirmed by identification tests.

If there is no growth of such type of colonies, or identification tests are negative it indicates absence of *P. aeruginosa* and the product passes the test.

Staphylococcus aureus

Using Casein soyabean digest broth as a diluent make 1 in 10 dilution of more than 1 g of the product as mentioned in *Total aerobic viable count* under *Microbial contamination in nonsterile products* (2.2.9) and use 10 ml or the quantity corresponding to 1 g or 1 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method*) of Casein soyabean digest broth incubate at 30° to 35° for 18 to 24 hours.

Sub-culture on a plate of Mannitol salt agar (Medium 14) incubate at 30° to 35° for 18 to 72 hours. Yellow or white colonies with yellow zones indicate the possibility of presence of *S.aureus*. This should be confirmed by identification tests.

If there is no growth of such type of colonies, or the identification tests are negative it indicates absence of *S. aureus* and the product passes the test.

Clostridia

Prepare a sample from the product under examination as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9). Take two equal portions corresponding to 1 g or 1 ml of the product and heat one portion 80° for 10 minutes and cool rapidly. Do not heat the other portion. Transfer 10 ml of each of the homogenised portions to two containers containing 100 ml of Reinforced medium for *Clostridia* (Medium 15). Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make sub-culture from each container on Columbia agar. (Medium 16) and incubate under anaerobic conditions at 30° to 35° for 48 hours.

The occurence of anaerobic growth of Gram positive bacilli with or without endospores giving a negative catalase test indicates possibility of presence of *Clostridia*.

If no anaerobic growth of microorganisms is detected on Columbia agar or identification test is negative, it indicates absence of *Clostridia* and the product passes the test.

Candida albicans

Prepare a sample from the product to be examined as mentioned in *Total aerobic viable count* under *Microbial contamination* (2.2.9) *in nonsterile products* and use the quantity corresponding to 1 g or 1 ml of the product to inoculate a suitable amount (determined as under *Validity of the Test method)* of Sabouraud dextrose broth (Medium 3) and incubate at 30° to 35° for 3 to 5 days.

Subculture on a plate of Sabouraud dextrose agar with antibiotic (Medium 4) and incubate at 30° to 35° for 24 to 48 hours. Growth of cream coloured colonies may indicate the possibility of presence of *C. albicans*. This is confirmed by identification tests.

If such colonies are not present, or the identification tests are negative, *C. albicans* is absent and the product passes the test.

Recommended solutions and culture media

The following solutions and culture media have been found to be satisfactory for the purpose for which they are prescribed in the tests for microbial contamination in the Pharmacopoeia. Other media may be used provided their suitability can be demonstrated.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 ml

0.1 per cent to 1.0 per cent w/v polysorbate 20 or polysorbate 80 may be added, if required. Sterilise.

Medium 1. Casein soyabean digest broth

Pancreatic digest of casein	17.0 g
Papaic digest of soyabean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Dextrose monohydrate	2.5 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2.

Medium 2. Casein soyabean digest agar

Pancreatic digest of casein	15.0 g
Papaic digest of soyabean meal	5.0 g

Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2.

Medium 3. Sabouraud dextrose broth

Peptones (meat and casein)	10.0 g
Dextrose monohydrate	20.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 5.6 ± 0.2.

Medium 4. Sabouraud dextrose agar with antibiotic(s)

Peptones (meat and casein)	10.0 g
Dextrose monohydrate	40.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 5.6 ± 0.2. Sterilise immediately before use, add 0.1 g of benzylpenicillin sodium and 0.1 g of tetracycline or alternatively add 50 mg of chloramphenicol per litre of medium as sterile solutions.

Medium 5. Enterobacteria enrichment broth-Mossel

Pancreatic digest of gelatin	10.0 g
Dextrose monohydrate	5.0 g
Dehydrated ox bile	20.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate dihydrate	8.0 g
Brilliant green	15.0 mg
Purified water	1000 ml

Adjust the pH after heating it is 7.2 ± 0.2. Heat at 100° for 30 minutes and cool immediately.

Medium 6. Violet red bile glucose agar

Yeast extract	3.0 g
Pancreatic digest of gelatin	7.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Dextrose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30.0 mg
Crystal violet	2.0 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°. Heat to boiling do not heat in an autoclave.

Medium 7. MacConkey broth

Pancreatic digest of gelatin	20.0 g
Lactose	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10.0 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2.

Medium 8. MacConkey agar

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein, equal parts)	3.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30.0 mg
Crystal violet	1.0 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.1 ± 0.2. Boil for 1 minute to effect solution.

Medium 9. Rappaport Vassiliadis Salmonella Enrichment broth

Soya peptone	4.5 g
Magnesium chloride hexahydrate	29.0 g
Sodium chloride	8.0 g
Dipotassium phosphate	0.4 g
Potassium dihydrogen phosphate	0.6 g
Malachite green	0.036 g
Purified water	1000 ml

Dissolve, warm slightly. Sterilise at 115° for 30 minutes pH 5.2 ± 0.2 after autoclaving.

Medium 10. Wilson and Blair's BBS Agar

Solution (i) Bismuth Sulphite Glucose Phosphate mixture

Bismuth ammonio - citrate scales	30.0 g
Sodium sulphite	100.0 g
Disodium hydrogen phosphate	100.0 g
Glucose	50.0 g
Purified water	1000 ml

Aseptically dissolve the Bismuth Ammonio – Citrate Scales in 250 ml of boiling water and the Sodium Sulphite in 500 ml of boiling water. Mix the solutions and while mixture is boiling add the Disodium Hydrogen Phosphate. When the mixture is cool, add the glucose previously dissolved in 250 ml boiling water and cool. This mixture can then be kept for few months.

Solution (ii) Iron citrate brilliant green mixture

Ferric citrate, brown scales	2.0 g
Brilliant green	0.25 g
Purified water	225 ml

With sterile precautions mix a solution of ferric citrate in 200 ml water with the solution of brilliant green in 25 ml water. This mixture can then be kept for few months.

Solution (iii) Nutrient Agar Solution

Peptone	1.0 g
Beef Extract	1.0 g
Sodium Chloride	0.5 g
Agar	2.0 g
Purified water	100 ml

Mix thoroughly, adjust the pH so that after sterilization it is 7.4 ± 0.2 and cool to 45° to 50°.

Complete Medium

Sterile Nutrient agar	100 ml
Bismuth sulphite glucose phosphate mixture	20 ml
Iron citrate brilliant green mixture	4.5 ml

Mix aseptically 20 volume of solution (i) and 4.5 volume of solution (ii) with 100 volume of solution (iii) previously melted of Sterile Nutrient agar and cool to a temperature 60° and pour.

Medium 11. GN Broth

Polypeptone peptone	20.0 g
Glucose	1.0 g
Sodium citrate	2.0 g
Sodium deoxycholate	0.5 g
Di-potassium hydrogen phosphate	4.0 g
Mono potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
Purified water	1000 ml

Adjust the pH so that after heating it is 7.0 ± 0.2. Mix and allow to stand for 15 minutes. With continuous stirring, bring gently to the boil and maintain at boiling point until solution is complete.

Medium 12. Xylose-Lysine-Desoxycholate agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80.0 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	800 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.4 ± 0.2. Heat just to boiling, cool to 50° and pour into petri dishes. Do not heat in an autoclave.

Medium 13. Cetrimide agar

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g
Potassium sulphate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Glycerin	10.0 g
Purified water	1000 ml

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilisation it is 7.0 to 7.4.

Medium 14. Mannitol Salt Agar Medium

Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Beef extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	25.0 mg
Purified water	1000 ml

Mix. Heat with frequent agitation and boil for 1 minute to effect solution. Adjust the pH after sterilisation to 7.4 ± 0.2.

Medium 15. Reinforced medium for Clostridia

Beef extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Dextrose monohydrate	5.0 g
Cysteine hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g
Purified water	1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is about 6.8 ± 0.2.

Medium 16. Columbia agar

Pancreatic digest of casein	10.0 g
Meat peptic digest	5.0 g
Heart pancreatic digest	3.0 g
Yeast extract	5.0 g
Maize starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0 g to 15.0 g
Purified water	1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise, allow to cool to 45° to 50°; add, where necessary, Gentamicin sulphate corresponding to 20 mg of gentamicin base and pour into petri dishes.

Acceptance Criteria for Microbiological Quality of Non Sterile Pharmaceutical Substances and Non Sterile Doses Forms

This provides acceptance criteria for microbiological quality of non sterile substances of pharmaceutical use and non sterile dosage forms, unless otherwise specified in the monograph.

If microorganisms are present in a pharmaceutical preparation, they can reduce or inactivate the therapeutic activity of the product or can adversely affect the health of the patient. Hence pharmaceutical preparations should have low bio-burden and they should not have specified microorganisms which are harmful.

Microbial examination of non-sterile products is performed according to the methods given above. Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use and non sterile pharmaceutical products based upon the Total Aerobic Viable Count (TAC) and the Total Fungal Count (TFC) are given in Tables 5 and 6 respectively.

The significance of microorganisms in nonsterile pharmaceutical products should be evaluated in terms of the use of the product, the nature of the product and the potential hazard to the user. This includes the list of specified microorganisms for which acceptance criteria are set. This list

Table 5 – Acceptance criteria for microbiological quality of nonsterile substances for pharmaceutical use

	TAC (CFU per g or per ml)	TFC (CFU per g or per ml)
Substances for pharmaceutical use	10^3	10^2

Table 6 – Acceptance criteria for microbiological quality of nonsterile dosage forms

Route of administration	TAC (CFU per g or per ml)	TFC (CFU per g or per ml)	Specified microorganisms
Non-aqueous preparation for oral use	10^2	10^2	*Escherichia coli* – absent in 1 g or 1ml
Aqueous preparation for oral use	10^2	10^1	*Escherichia coli* – absent in 1 g or 1ml
Rectal use	10^3	10^2	---
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	*Staphylococcus aureus* – absent in 1 g or 1 ml *Pseudomonas aeruginosa* – absent in 1 g or 1 ml
Vaginal use	10^2	10^1	*Staphylococcus aureus* – absent in 1 g or 1 ml *Pseudomonas aeruginosa* – absent in 1 g or 1 ml *Candida albicans* – absent in 1 g or 1 ml
Inhalation use (special requirements apply to Liquid preparations for nebulisation)	10^2	10^1	*Staphylococcus aureus* – absent in 1 g or 1 ml *Pseudomonas aeruginosa* – absent in 1 g or 1 ml Bile-tolerant Gram-negagive bacteria –absent 1 g or 1 ml
Transdermal patches (limits for one patch including adhesive layers and backing)	10^2	10^1	*Staphylococcus aureus* – absent in 1 g or 1 ml *Pseudomonas aeruginosa* – absent in 1 g or 1 ml

is not exhaustive and depending on the nature of the ingredients and manufacturing process it may be necessary to test for other microorganisms. The importance of the microorganisms isolated from the product may be evaluated in terms of the following.

i. The nature of the product, its ability to support growth, the type and the quantity of the antimicrobial preservatives added.
ii. Use of product. Hazard varies according to the place of application (on intact skin or on wound) as well as route of administration (eye, nose, oral).
iii. Use of immunosuppressive agents, corticosteroids, etc.
iv. Intended recipient. Risk may vary for neonates, adults and the debilitated.

"Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality."

Acceptance Criteria for Microbiological Quality of Herbal Medicinal Products for Oral Use

This provides acceptance criteria for microbiological quality of herbal medicinal products for oral use, unless otherwise specified in the monograph. The significance of microorganisms in herbal products should be evaluated in terms of the use of the product, the nature of the product and the potential hazard to the user. This includes the list of specified microorganisms for which acceptance criteria are set. This list is not exhaustive and depending on the nature of the ingredients and manufacturing process it may be necessary to test for other microorganisms.

Microbial examination of non sterile products is performed according to the methods given above. Acceptance criteria for microbiological quality of herbal medicinal products for oral use based upon the Total Aerobic Viable Count (TAC) and the Total Fungal Count (TFC) are given in Table 7A, 7B and 7C respectively. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed.

Table 7 A – Herbal medicinal products containing herbal drugs, with or without excipients, intended for the preparation of infusions and decoctions using boiling water (for example herbal teas, with or without added flavourings).

TAC	Acceptance criterion: 10^7 CFU per g
TFC	Acceptance criterion: 10^5 CFU per g
Escherichia coli	Acceptance criterion: 10^3 CFU per g
Salmonella	Absent in 10 g
Shigella	Absent in 10 g

Table 7 B – Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where the method of processing (for example, extraction) or, where appropriate, in the case of herbal drugs, of pre-treatment reduces the levels of organisms to below those stated for this category.

TAC	Acceptance criterion: 10^4 CFU per g or CFU per ml
TFC	Acceptance criterion: 10^2 CFU per g or CFU per ml
Bile-tolerant Gram-negative bacteria	Acceptance criterion: 10^2 CFU per g or CFU per ml
Escherichia coli	Absent in 1 g or 1 ml
Salmonella	Absent in 10 g or 10 ml
Shigella	Absent in 10 g or 10 ml

Table 7 C – Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where it can be demonstrated that the method of processing (for example, extraction with low strength ethanol or water that is not boiling or low temperature concentration) or, in the case of herbal drugs, of pre-treatment, would not reduce the level of organisms sufficiently to reach the criteria required under 7 B.

TAC	Acceptance criterion: 10^5 CFU per g or CFU per ml
TFC	Acceptance criterion: 10^4 CFU per g or CFU per ml
Bile-tolerant Gram-negative bacteria	Acceptance criterion: 10^4 CFU per g or CFU per ml
Escherichia coli	Absent in 1 g or 1 ml
Salmonella	Absent in 10 g or 10 ml
Shigella	Absent in 10 g or 10 ml

It is recognized that for some herbal medicinal products the criteria given above under 7A, 7B or 7C for TAC, TFC and bile tolerant gram-negative bacteria can not be met because of the typical level of microbial contamination. Higher acceptance criteria may be applied on the basis of risk assessment that takes account of qualitative and quantitative characterization of the bioburden and the intended use of the medicinal product.

2.2.10. Microbiological Assay of Antibiotics

The microbiological assay of an antibiotic is based upon a comparison of the inhibition of growth of microorganisms by measured concentrations of the antibiotics under examination with that produced by known concentrations of a standard

preparation of the antibiotic having a known activity. Two general methods are usually employed, the cylinder-plate (or cup-plate) method and the turbidimetric (or tube assay) method.

The cylinder-plate method (Method A) depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic. The Turbidimetric method (Method B) depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favourable to its rapid growth in the absence of the antibiotic.

The assay is designed in such a way that the mathematical model on which the potency equation is based can be proved to be valid. If a parallel-line model is chosen, the two log dose-response lines of the preparation under examination and the standard preparation should be parallel; they should be rectilinear over the range of doses used in the calculation. These conditions should be verified by validity tests for a given probability. Other mathematical models, such as the slope ratio method, may be used provided that proof of validity is demonstrated.

Media. Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 1. Minor modifications of the individual ingredients may be made, or reconstituted dehydrated media may be used provided the resulting media have equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in sufficient *water* to produce 1000 ml and add sufficient *1 M sodium hydroxide* or *1 M hydrochloric acid*, as required so that after sterilisation the pH is as given in Table 1.

Standard Preparation and Units of Activity

A Standard Preparation is an authentic sample of the appropriate antibiotic for which the potency has been precisely determined by reference to the appropriate international standard. The potency of the standard preparation may be expressed in International Units or in µg per mg of the pure antibiotic.

The Standard Preparations are certified by the laboratory of the Indian Pharmacopoeia Commission or by any other notified laboratory(ies) and are maintained and distributed by the agency(ies) notified for the purpose.

A Standard Preparation may be replaced by a working standard prepared by any laboratory which should be compared at definite intervals under varying conditions with the standard.

Buffer Solutions. Prepare by dissolving the following quantities given in Table 2 of *dipotassium hydrogen phosphate* and *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml after sterilisation, adjusting the pH with *8 M phosphoric acid* or *10 M potassium hydroxide*.

Table 1– Media: Quantities in g of ingredients per 1000 ml

Ingredient	Medium									
	A	B	C	D	E	F	G	H	I	J
Peptone	6.0	6.0	5.0	6.0	6.0	6.0	9.4	–	10.0	–
Pancreatic digest of casein	4.0	–	–	4.0	–	–	–	17.0	–	15.0
Yeast extract	3.0	3.0	1.5	3.0	3.0	3.0	4.7	–	–	–
Beef extract	1.5	1.5	1.5	1.5	1.5	1.5	2.4	–	10.0	–
Dextrose	1.0	–	1.0	1.0	–	–	10.0	2.5	–	–
Papaic digest of soyabean	–	–	–	–	–	–	–	3.0	–	5.0
Agar	15.0	15.0		15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerin	–	–	–	–	–	–	–	–	10.0	–
Polysorbate 80	–	–	–	–	–	–	–	10.0*	–	–
Sodium chloride	–	–	3.5	–	–	–	10.0	5.0	3.0	5.0
Di-potassium hydrogen phosphate	–	–	3.68	–	–	–	–	2.5	–	–
Potassium dihydrogen phosphate	–	–	1.32	–	–	–	–	–	–	–
Final pH (after sterilisation)	6.5 - 6.6	6.5 - 6.6	6.95 - 7.05	7.8 - 8.0	7.8 - 8.0	5.8 - 6.0	6.0 - 6.2	7.1 - 7.3	6.9 - 7.1	7.2 - 7.4

* Quantity in ml, to be added after boiling the media to dissolve the agar.

Table 3 - Stock solutions and test dilutions of Standard Preparation

Antibiotic	Standard Stock Solution					Test Dilution		
	Assay Method	Prior Drying	Initial solvent (further diluent, if different)	Final Stock Concentration per ml	Use before (number of days)	Final diluent	Median dose µg or Units per ml	Incubation temp (°)
Amikacin	B	No	Water	1 mg	14	Water	10 µg	32 - 35
Amphotericin B	A	Yes	DMSO[11]	1 mg	Same day	B5	1.0 µg	29 - 31
Bacitracin	A	Yes	0.01M HCl	100 units	Same day	B1	1.0 Unit	32 - 35
Bleomycin	A	Yes	B6[8]	2 units	14	B6	0.04 Unit	32 - 35
Capreomycin	B	Yes	Water	1 mg	7	Water	100 µg	36 - 37.5
Carbenicillin	A	No	B1	1 mg	14	B6	20 µg	36 - 37.5
Chlortetracycline	A[1]	No	0.1M HCl	1 mg	4	Water	2.5 µg	37 - 39
	B[10]	No	0.1M HCl	1 mg	4	Water	0.24 µg	35 - 37
Colistimethate sodium	A	Yes	Water	12700 units	Same day	B4	1.0 Unit	35 - 39
	B[14]	Yes	Water	12700 units	Same day	B6	1.0 Unit	35 - 37
Colistin sulphate	A	No	Water	1 mg	14	B6	1.0 µg	35 - 37
Erythromycin	A	Yes	Methanol (10 mg / ml)[6] (B2)	1 mg	14	B2	1.0 µg	32 - 35
Framycetin	A	Yes	B2	1 mg	14	B2	1.0 µg	30 - 37
	B[13]	Yes	B2	1 mg	14	B2	10 µg	35 - 37
Gentamicin	A	Yes	B2	1 mg	30	B2	0.1 µg	36 - 37.5
	B[13]	Yes	Water	1 mg	30	B6	0.5 µg	35 - 37
Gramicidin	B	Yes	Methanol	1000 units	30	B6[12]	1.0 Unit	35 - 37
Kanamycin sulphate	A[1]	No	B2	800 units	30	B2	0.8 Unit	37 - 39
	B[2]	No	Water	1000 units	30	Water	10 Units	32 - 35
Neomycin	A	Yes	B2	1 mg	14	B2	1.0 µg	36 - 37.5
	B[13]	Yes	Water	1 mg	14	B2	1.0 µg	35 - 37
Netilmicin sulphate	A	Yes	Water	1 mg	7	B2	0.2 µg	32 - 35
Novobiocin	A	Yes	Ethanol (10 mg/ml)[9], (B2)	1 mg	5	B4	0.5 µg	34 - 36
Nystatin	A	Yes	DMF[7]	1000 units	Same day	B4	20 Units	29 - 31
Oxytetracycline	A[3]	No	0.1M HCl	1 mg	4	B3	2.5 µg	32 - 35
	B[2]	No	0.1M HCl	1 mg	4	Water	0.24 µg	35 - 37
Polymyxin B	A	Yes	Water, (B4)	10000 units	14	B4	10 Units	35 - 39
Sisomicin	A	Yes	B2	1 mg	14	B2	0.1 µg	32 - 35
Spiramycin	A[4]	No	Methanol	3200 units	1	B2	20 Units	30 - 32
	B[13]	No	Methanol	3200 units	1	B6	50 Units	35 - 37
Streptomycin	A[4]	Yes	Water	1 mg	30	Water	1.0 µg	32 - 35
	B[5]	Yes	Water	1 mg	30	Water	30 µg	35 - 37
Teicoplanin	A	Yes	B4	1000 units	Same day	B4	1.0 Unit	35 - 37
Tetracycline	A[3]	No	0.1M HCl	1 mg	1	Water	2.5 µg	32 - 35
	B[6]	No	0.1M HCl	1 mg	1	Water	0.24 µg	35 - 37
Tobramycin	B	Yes	Water	1 mg	14	Water	2.5 µg	32 - 35

Tylosin	A	No	Methanol (2.5 per cent v/v)[9] in B6	1000 units	30	40 volumes of Methanol and 60 volumes of B2	0.25 Unit	32-35
	B[10]	No	Methanol	1000 units	30	Methanol and B2 (1:1)	4 Units	36-38
Tyrothricin	B	Yes	Ethanol	1 mg	Same Day	Ethanol	1.0 Unit	36-38
Vancomycin	A	No	Water	1 mg	7	B3	10 µg	32-35
	B[13]	No	Water	1 mg	7	B2	10 µg	37-39

1. With *Bacillus pumilus* ATCC 14884 as test organism;
2. With *Staphylococcus aureus* ATCC 29737 as test organism;
3. With *Bacillus cereus var mycoides* ATCC 11778 as test organism;
4. With *Bacillus subtilis* ATCC 6633 as test organism;
5. With *Klebsiella pneumoniae* ATCC 10031 as test organism;
6. With *Staphylococcus aureus* ATCC 29737 as test organism;
7. DMF = Dimethylformamide;
8. In columns 4 & 7, B denotes buffer solution and the number following refers to the buffer number in Table 2;
9. Initial concentration of stock solution;
10. With *Staphylococcus aureus* ATCC 9144 as test organism;
11. DMSO: Dimethyl sulphoxide;
12. Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions for example 0.1 mg per ml of polysorbate 80;
13. With *Staphylococcus aureus* ATCC 6538P as test organism.
14. With *Escherichia coli* ATCC 9637 as test organism.

NOTES — For Amphotericin B and Nystatin, prepare the standard solutions and the sample test solution simultaneously.

For Amphotericin B, further dilute the stock solution with dimethyl sulphoxide to give concentrations of 12.8, 16, 20, 25 and 31.2 µg per ml prior to making the test solutions. The test dilution of the sample prepared from the solution of the substance under examination should contain the same amount of dimethyl sulphoxide as the test dilutions of the Standard Preparation.

For Bacitracin, each of the standard test dilutions should contain the same amount of hydrochloric acid as the test dilution of the sample.

For Nystatin, further dilute the stock solution with dimethylformamide to give concentrations of 64.0, 80.0, 100.0, 125.0, 156.0 µg per ml prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilution of the sample being examined. The test dilution of the sample prepared from the solution of the substance under examination should contain the same amount of dimethylformamide as test dilutions of the Standard Preparation. Protect the solutions from light.

When making the stock solution of Polymyxin B, add 2 ml of water for each 5 mg of the weighted Standard Preparation material.

Where indicated, dry about 100 mg of the Standard Preparation before use in an oven at a pressure not exceeding 0.7 kPa at 60° for 3 hours, except in the case of Bleomycin (dry at 25° for 4 hours), Novobiocin (dry at 100° for 4 hours), Gentamicin (dry at 110° for 3 hours) and Nystatin (dry at 40° for 2 hours).

Where two level factorial assays are performed use the following test doses per ml: Amphotericin B, 1.0 to 4.0 µg; Bacitracin, 1.0 to 4.0 units; Kanamycin sulphate, 5.0 to 20.0 units; Streptomycin, 5.0 to 20.0 µg.

Table 2 – Buffer Solutions

Buffer No.	Dipotassium hydrogen phospahate, K_2HPO_4 (g)	Potassium dihydrogen phosphate, KH_2PO_4 (g)	pH adjusted after sterilisation to
1	2.0	8.0	6.0±0.1
2	16.73	0.523	8.0±0.1
3	–	13.61	4.5±0.1
4	20.0	80.00	6.0±0.1
5	35.0	–	10.5±0.1*
6	13.6	4.0	7.0±0.2

* *After addition of 2 ml of 10M potassium hydroxide*

Preparation of the Standard Solution. To prepare a stock solution, dissolve a quantity of the Standard Preparation of a given antibiotic, accurately weighed and previously dried where so indicated in Table 3, in the solvent specified in the table, and then dilute to the required concentration as indicated. Store in a refrigerator and use within the period indicated. On the day of assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio 1:1.25 for Method A or smaller for Method B. Use the final diluent specified and a sequence such that the middle or median has the concentration specified in Table 3.

Preparation of the Sample Solution: From the information available for the substance under examination (the "unknown"), assign to it an assumed potency per unit weight

or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic in Table 3 but with the same final diluent as used for the Standard Preparation. The assay with 5 levels of the Standard requires only one level of the unknown at a concentration assumed equal to the median level of the standard.

Test Organisms. The test organism for each antibiotic is listed in Table 4, together with its identification number in the American Type Culture Collection (ATCC). Maintain a culture on slants of the medium and under the incubation conditions specified in Table 5, and transfer weekly to fresh slants.

Table 4 - Test Organisms for Microbiological Assay of Antibiotics

Antibiotic	Test Organism	ATCC[1] No.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Capreomycin	*Klebsiella pneumoniae*	10031
Carbenicillin	*Pseudomonas aeruginosa*	25619
Chlortetracycline	*Bacillus pumilus*	14884
	Staphylococcus aureus	9144
Colistimethate-sodium	*Bordetella bronchiseptica*	4617
	Escherichia coli	10536
	Escherichia coli	9637
Colistin sulphate	*Bordetella bronchiseptica*	4617
	Escherichia coli	10536
Erythromycin	*Kocuria rhizophila*	9341
Framycetin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
	Staphylococcus aureus	6538P
Gentamicin	*Bacillus pumilus*	14884
	Staphylococcus aureus	6538P
	Staphylococcus epidermidis	12228
Gramicidin	*Enterococcus hirae*	10541
	Staphylococcus aureus	6538P
Kanamycin sulphate	*Bacillus pumilus*	14884
	Staphylococcus aureus	29737
Neomycin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
	Staphylococcus aureus	6538P
	Staphylococcus epidermidis	12228
Netilmicin sulphate	*Staphylococcus aureus*	6538P
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Bacillus cereus var. mycoides*	11778
	Staphylococcus aureus	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Sisomicin	*Staphylococcus epidermidis*	12228
Spiramycin	*Bacillus subtilis*	6633
	Staphylococcus aureus	6538P
Streptomycin	*Bacillus subtilis*	6633
	Klebsiella pneumoniae	10031
Teicoplanin	*Bacillus subtilis*	6633
Tetracycline	*Bacillus cereus*	11778
	Staphylococcus aureus	29737
Tobramycin	*Staphylococcus aureus*	29737
Tylosin	*Kocuria rhizophila*	9341
	Staphylococcus aureus	9144
Tyrothricin	*Enterococcus hirae*	10541
Vancomycin	*Bacillus subtilis*	6633
	Staphylococcus aureus	6538P

1. American Type Culture Collection, 21301 Park Lawn Drive, Rockville, MD20852, USA.

Preparation of inoculum. Prepare the microbial suspensions for the inoculum for the assay as given in Table 5. If the suspensions are prepared by these methods, growth characteristics are sufficiently uniform so that the inoculum can be adequately determined by the trials given below.

Determination of inoculum

For Method A. After the suspension is prepared as given under Table 5, add different volumes of it to each of several different flasks containing 100 ml of the medium specified in Table 5 (the volume of suspension suggested in Table 5 may be used as a guide). Using these inocula, prepare inoculated plates as described for the specific antibiotic assay. While conducting cylinder-plate assays, double-layer plates may be prepared by pouring a seed layer (inoculated with the desired micro organism) over a solidified uninoculated base layer. For each Petri dish, 21 ml of base layer and 4 ml of the seed layer may be generally suitable. Fill each cylinder with the median concentration of the antibiotic (Table 3) and then incubate the plates. After incubation, examine and measure the zones of inhibition. The volume of suspension that produces the optimum zones of inhibition with respect to both clarity and diameter determines the inoculum to be used for the assay.

For Method B. Proceed as described for Method A and, using the several inocula, carry out the procedure as described for the specific antibiotic assay running only the high and low concentrations of the standard response curve. After incubation, read the absorbances of the appropriate tubes. Determine which inoculum produces the best response between the low and high antibiotic concentrations and use this inoculum for the assay.

Table 5 – Preparation of inoculum

Test organism	Incubation conditions			Suggested dilution factor	Suggested inoculum composition		
	Medium/ Method of Preparation	Temp. (°)	Time		Medium	Amount (ml per 100 ml)	Antibiotics assayed
Bacillus cereus var. mycoides(11778)	A[1] /2	32-35	5 days	-	F	As required	Oxytetracycline
							Tetracycline
Bacillus pumilus (14884)	A[1] /2	32-35	5 days	-	D	As required	Chlortetracycline
							Framycetin
							Gentamicin
							Kanamycin sulphate
							Neomycin
Bacillus subtilis (6633)	A[1] /2	32-35	5 days	-	E	As required	Framycetin
					E	As required	Kanamycin B
					D	As required	Neomycin
					B	As required	Spiramycin
					A	As required	Streptomycin
					E	As required	Teicoplanin
					A	As required	Vancomycin
Bordetella bronchiseptica (4617)	A/1	32-35	24 hr	1:20	H	0.1	Colistimethate sodium
					H	0.1	Colistin sulphate
					H	0.1	Polymyxin B
Escherichia coli (10536)	A/1	35-39	24 hr	1:20	H	0.1	Colistimethate sodium
					H	0.1	Colistin sulphate
Escherichia coli (9637)	A/1	35-37	24 hr	-	C	0.1	Colistimethate sodium
Enterococcus hirae (10541)	A/1	36-38	16-18 hr	-	C	1.0	Gramicidin
					C	1.0	Tyrothricin
Klebsiella pneumoniae (10031)	A/1	36-37	24 hr	1:25	C	0.1	Capreomycin
							Streptomycin
Kocuria rhizophila (9341)	A/1	32-35	24 hr	1:40	D	1.5	Erythromycin
					D	1.2	Tylosin
Micrococcus luteus (10240)	A/1	32-35	24 hr	1:35	A	0.3	Bacitracin
Mycobacterium smegmatis (607)	J/4	36-37.5	48 hr	As determined	I	1.0	Bleomycin
Pseudomonas aeruginosa (25619)[2]	A/1	36-37.5	24 hr	1:25	H	0.5	Carbenicillin
Saccharomyces cerevisiae (9763)	G/3	29-31	48 hr	As determined	G	1.0	Amphotericin B
Saccharomyces cerevisiae (2601)	G/3	29-31	48 hr	As determined	G	1.0	Nystatin
Staphylococcus aureus (6538P)	A/1	32-35	16-18 hr	-	C	3.0	Framycetin
			24 hr	-	C	1.0	Gentamicin
			16-18 hr	-	C	1.0	Gramicidin
			16-18 hr	-	C	0.5	Neomycin
			24 hr	-	A	1.0	Netilmicin sulphate
			16-18 hr	-	C	0.5	Spiramycin
	A/1	37-39	24 hr	-	C	As required	Vancomycin

Test organism	Incubation conditions			Suggested	Suggested inoculum composition		
	Medium/ Method of Preparation	Temp. (°)	Time	dilution factor	Medium	Amount (ml per 100 ml)	Antibiotics assayed
Staphylococcus aureus (29737)	A/1	32-35	24 hr	1:20	C	0.1	Amikacin Doxycycline Oxytetracycline Tetracycline Tobramycin
					C	0.2	Kanamycin sulphate
Staphylococcus aureus (9144)	A/1	35-37	16-18 hr	-	C	0.25	Tylosin Chlortetracycline
Staphylococcus epidermidis (12228)	A/1	32-35	24 hr	1:40	D	0.03	Gentamicin
					D	0.4	Neomycin
					A	4.0	Novobiocin
					D	0.03	Sisomicin

1. Use Medium A containing 300 mg of manganese sulphate per litre.
2. For *Pseudomonas aeruginosa* in the assay of Carbenicillin, use the dilution yielding 25 per cent light transmission, rather than the stock suspension, for preparing the inoculum suspension.

Methods of preparation of test organism suspension:

1. Maintain the test organism on slants of Medium A and transfer to a fresh slant once a week. Incubate the slants at the temperature indicated above for 24 hours. Using 3 ml of *saline solution*, wash the organism from the agar slant onto a large agar surface of Medium A such as a Roux bottle containing 250 ml of agar. Incubate for 24 hours at the appropriate temperature. Wash the growth from the nutrient surface using 50 ml of *saline solution*. Store the test organism under refrigeration. Determine the dilution factor which will give 25 per cent light transmission at about 530 nm. Determine the amount of suspensions to be added to each 100 ml of agar of nutrient broth by use of test plates or test broth. Store the suspension uder refrigeration.
2. Proceed as described in Method 1 but incubate the Roux bottle for 5 days. Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 to 70 ml of saline solution and heat the suspension for 30 minutes at 70°. Wash the spore suspension three times with 50 to 70 ml of *saline solution*. Resuspend in 50 to 70 ml of *saline solution* and heat- shock again for 30 minutes. Use test plates to determine the amount of the suspension required for 100 ml of agar. Store the suspension under refrigeration.
3. Maintain the test organism on 10 ml agar slants of Medium G. Incubate at 32° to 35° for 24 hours. Inoculate 100 ml of nutrient broth. Incubate for 16 to 18 hours at 37° and proceed as described in Method 1.
4. Proceed as described in Method 1 but wash the growth from the nutrient surface using 50 ml of Medium 1 (prepared without agar) in place of *saline solution*.

Apparatus

All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms is sterilised by dry heat or by steam.

Temperature Control. Thermostatic control is required at several stages of a microbial assay, when culturing a micro-organism and preparing its inoculum and during incubation in a plate assay. Closer control of the temperature is imperative during incubation in a tube assay which may be achieved by either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.

Spectrophotometer. Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580 nm filter for preparing inocula of the required density or with a 530 nm filter for reading a absorbance in a tube assay. For the latter purpose, the instrument may be arranged to accept the tube in which incubation takes place, to accept a modified cell fitted with a drain that facilitates rapid change of contents, or preferably fixed with a flow-through cell for a continuous flow-through analysis. Set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample.

Cylinder-plate Assay Receptacles. Use rectangular glass trays or glass or plastic petri dishes (approximately 20 x 100 mm) having covers of suitable material and assay cylinders made of glass, porcelain, aluminium or stainless steel with outside diameter 8 mm ± 0.1 mm, inside diameter 6 mm ± 0.1 mm and

length 10 mm ± 0.1 mm. Instead of cylinders, holes 5 to 8 mm in diameter may be bored in the medium with a sterile borer, or paper discs of suitable quality paper may be used. Carefully clean the cylinder to remove all residues. An occasional acid-bath, e.g. with about *2 M nitric acid* or with *chromic acid solution* is needed.

Turbidimetric Assay Receptacles. For assay tubes, use glass or plastic test-tubes, e.g. 16 mm x 125 mm or 18 mm x 150 mm that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. Cleanse thoroughly to remove all antibiotic residues and traces of cleaning solution and sterilise tubes that have been used previously before subsequent use.

Assay Designs

Microbial assays gain markedly in precision by the segregation of relatively large sources of potential error and bias through suitable experimental designs. In a cylinder plate assay, the essential comparisons are restricted to relationships between zone diameter measurements within plates, exclusive of the variation between plates in their preparation and subsequent handling. To conduct a turbidimetric assay so that the difference in observed turbidity will reflect the differences in the antibiotic concentration requires both greater uniformity in the environment created for the tubes through closer thermostatic control of the incubator and the avoidance of systematic bias by a random placement of replicate tubes in separate tube racks, each rack containing one complete set of treatments. The essential comparisons are then restricted to relationships between the observed turbidities within racks.

Within these restrictions, two alternative designs are recommended; i.e. a 3-level (or 2-level) factorial assay, or a 1-level assay with a standard curve. For a factorial assay, prepare solutions of 3 or 2 corresponding test dilutions for both the standard and the unknowns on the day of the assay, as described under Preparation of the Standard and Preparation of the samples. For a 1-level assay with a standard curve, prepare instead solutions of five test dilutions of the standard and a solution of a single median test level of the unknown as described in the same sections. Consider an assay as preliminary if its computed potency with either design is less than 60 per cent or more than 150 per cent of that assumed in preparing the stock solution of the unknown. In such a case, adjust its assumed potency accordingly and repeat the assay.

Microbial determinations of potency are subject to inter-assay variables as well as intra-assay variables, so that two or more independent assays are required for a reliable estimate of the potency of a given assay preparation or unknown. Starting with separately prepared stock solutions and test dilutions of both the standard and unknown, repeat the assay of a given unknown on a different day. If the estimated potency of the second assay differs significantly, as indicated by the calculated standard error, from that of the first, conduct one or more additional assays. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes.

Methods

Carry out the microbiological assay by Method A or Method B.

A. Cylinder-plate or Cup-plate method

Inoculate a previously liquified medium appropriate to the assay (Tables 1 and 3) with the requisite quantity of suspension of the micro organism, add the suspension to the medium at a temperature between 40° and 50° and immediately pour the inoculated medium into the petri dishes or large rectangular plates to give a depth of 3 to 4 mm (1 to 2 mm for nystatin). Ensure that the layers of medium are uniform in thickness, by placing the dishes or plates on a level surface.

Store the prepared dishes or plates in a manner so as to ensure that no significant growth or death of the test organism occurs before the dishes or plates are used and that the surface of the agar layer is dry at the time of use.

Using the appropriate buffer solutions indicated in Tables 2 and 3, prepare solutions of known concentrations of the standard preparation and solutions of the corresponding assumed concentrations of the antibiotic to be examined. Where directions have been given in the individual monograph for preparing the solutions, these should be followed and further dilutions made with buffer solution as indicated in Table 3. Apply the solutions to the surface of the solid medium in sterile cylinders or in cavities prepared in the agar. The volume of solution added to each cylinder or cavity must be uniform and sufficient almost to fill the holes when these are used. When paper discs are used these should be sterilised by exposure of both sides under a sterilising lamp and then impregnated with the standard solutions or the test solutions and placed on the surface of the medium. When petri dishes are used, arrange the solutions of the Standard Preparation and the antibiotic under examination on each dish so that, they alternate around the dish and so that the highest concentrations of standard and test preparations are not adjacent. When plates are used, place the solutions in a Latin square design, if the plate is a square, or if it is not, in a randomised block design. The same random design should not be used repeatedly.

Leave the dishes or plates standing for 1 to 4 hours at room temperature or at 4°, as appropriate, as a period of pre-incubation diffusion to minimise the effects of variation in time between the application of the different solutions. Incubate them for about 18 hours at the temperature indicated in Table 3. Accurately measure the diameters or areas of the circular inhibition zones and calculate the results.

Selection of the assay design should be based on the requirements stated in the individual monograph. Some of the usual assay designs are as follows.

(a) One-level Assay with Standard Curve

Standard Solution. Dissolve an accurately weighed quantity of the Standard Preparation of the antibiotic, previously dried where necessary, in the solvent specified in Table 3, and then dilute to the required concentration, as indicated, to give the stock solution. Store in a refrigerator and use within the period indicated. On the day of the assay prepare from the stock solution, 5 dilutions (solution S_1 to S_5) representing 5 test levels of the standard and increasing stepwise in the ratio of 5:4. Use the diluent specified in Table 3 and a sequence such that the middle or median has the concentration given in the table.

Sample Solution. From the information available for the antibiotic preparation which is being examined (the "unknown") assign to it an assumed potency per unit weight or volume and on this assumption prepare on the day of the assay a stock solution with same solvent as used for the standard. Prepare from this stock solution a dilution to a concentration equal to the median level of the standard to give the sample solution.

Method. For preparing the standard curve, use a total of 12 petri dishes or plates to accommodate 72 cylinders or cavities. A set of 3 plates (18 cylinders or cavities) is used for each dilution. On each of the three plates of a set fill alternate cylinders or cavities with solution S_3 (representing the median concentration of the standard solution) and each of the remaining 9 cylinders or cavities with one of the other 4 dilutions of the standard solution. Repeat the process for the other 3 dilutions of the standard solution. For each unknown preparation use a set of 3 plates (18 cylinders or cavities) and fill alternate cylinders or cavities with the sample solution and each of the remaining 9 cylinders of cavities with solution S_3.

Incubate the plates for about 18 hours at the specified temperature and measure the diameters or the zones of inhibition.

Estimation of Potency. Average the readings of solution S_3 and the readings of the concentration tested on each sets of three plates, and average also all 36 readings of solution S_3. The average of the 36 readings of solution S_3 is the correction point for the curve. Correct the average value obtained for each concentration (S_1, S_2, S_4 and S_5) to the figure it would be if the readings for solution S_3 for that set of three plates were the same as the correction point. Thus, in correcting the value obtained with any concentration, say S_1, if the average of 36 readings of S_3 is, for example, 18.0 mm and the average of the S_3 concentrations on one set of three plates is 17.8 mm, the correction is + 0.2 mm. If the average reading of S_1 is 16.0 mm the corrected reading of S_1 is 16.2 mm. Plot these corrected values including the average of the 36 readings for solutions S_3 on two-cycle semilog paper, using the concentrations in Units or mg per ml (as the ordinate logarithmic scale) and the diameter of the zones of inhibition as the abscissa. Draw the straight response line either through these points by inspection or through the points plotted for highest and lowest zone diameters obtained by means of the following expressions:

$$L = \frac{3a + 2b + c - e}{5}; \quad H = \frac{3e + 2d + c - a}{5}$$

where, L = the calculated zone diameter for the lowest concentration of the standard curve response line.

H = the calculated zone diameter for the highest concentration of the standard curve response line.

c = average zone diameter of 36 readings of the reference point standard solution.

a,b,d,e = corrected average values for the other standard solutions, lowest to highest concentrations, respectively.

Average the zone diameters for the sample solution and for solutions S_3 on the plates used for the sample solution. If sample gives a large average zone size than the average of the standard (solution S_3), add the difference between them to the zone size of solution S_3 of the standard response line. If the average sample zone size is smaller than the standard values, subtract the difference between them from the zone size of solution S_3 of the standard response line. From the response line read the concentration corresponding to these corrected values of zone sizes. From the dilution factors the potency of the sample may be calculated.

(b) Two-level Factorial Assay

Prepare parallel dilutions containing 2 levels of both the standard (S_1 and S_2) and the unknown (U_1 and U_2). On each of four or more plates, fill each of its four cylinders or cavities with a different test dilution, alternating standard and unknown. Keep the plates at room temperature and measure the diameters of the zones of inhibition.

Estimation of Potency. Sum the diameters of the zones of each dilution and calculate the percentage potency of the sample (in terms of the standard) from the following equation.:

$$\text{Per cent potency} = \text{Antilog}\,(2.0 + a \log I)$$

wherein a may have a positive or negative value and should be used algebraically and

$$\text{where } a = \frac{(U_1 + U_2) - (S_1 + S_2)}{(U_1 - U_2) + (S_1 - S_2)}$$

U_1 and U_2 = are the sums of the zone diameters with solutions of the unknown of high and low levels.

S_1 and S_2 = are the sums of the zone diameters with solutions of the standard of high and low levels.

I = ratio of dilutions.

If the potency of the sample is lower than 60 per cent or greater than 150 per cent of the standard, the assay is invalid and should be repeated using higher or lower dilutions of the same solution.

The potency of the sample may be calculated from the expression:

$$\frac{\text{per cent potency} \times \text{assumed potency of the sample}}{100}$$

(c) Other Designs

1. Factorial assay containing parallel dilution of three test levels of standard and the unknown.
2. Factorial assay using two test levels of standard and two test levels of two different unknowns.

B. Turbidimetric or Tube Assay Method

The method has the advantage of a shorter incubation period for the growth of the test organism (usually 3 to 4 hours) but the presence of solvent residues or other inhibitory substances affects this assay more than the cylinder plates assay and care should be taken to ensure freedom from such substances in the final test solutions. This method is not recommended for cloudy or turbid preparations.

Prepare five different concentrations of the standard solution for preparing the standard curve by diluting the stock solution of the Standard Preparation of the antibiotic (Table 3) and increasing stepwise in the ration 5:4. Select the median concentration (Table 3) and dilute the solution of the substance being examined (unknown) to obtain approximately this concentration. Place 1 ml of each concentration of the standard solution and of the sample solution in each of the tubes in duplicate. To each tube add 9 ml of nutrient medium (Table 3) previously seeded with the appropriate test organism (Table 3).

At the same time prepare three control tubes, one containing the inoculated culture medium (culture control), another identical with it but treated immediately with 0.5 ml of *dilute formaldehyde solution* (blank) and a third containing uninoculated culture medium.

Place all the tubes, randomly distributed or in a randomized block arrangement, in an incubator or water-bath and maintain them at the specified temperature (Table 3) for 3 to 4 hours. After incubation add 0.5 ml of *dilute formaldehyde solution* to each tube. Measure the growth of the test organism by determining the absorbance at about 530 nm of each of the solutions in the tubes against the blank (2.4.7).

Estimation of potency. Plot the average absorbances for each concentration of the standard on *semi-logarithmic* paper with the absorbances on the arithmetic scale and concentrations on the logarithmic scale. Construct the best straight response line through the points either by inspection or by means of the following expressions:

$$L = \frac{3a + 2b + c - e}{5}; \quad H = \frac{3e + 2d + c - a}{5}$$

where, L = the calculated absorbance for the lowest concentration of the standard response line.

H = the calculated absorbance for the highest concentration of the standard response line.

a, b, c, d, e = average absorbance values for each concentration of the standard response line lowest to highest respectively.

Plot the values obtained for L and H and connect the points. Average the absorbances for the sample and read the antibiotic concentration from the standard response line. Multiply the concentration by the appropriate dilution factors to obtain the antibiotic content of the sample.

Precision of Microbiological Assays

The fiducial limits of error of the estimated potency should be not less than 95 per cent and not more than 105 per cent of the estimated potency unless otherwise stated in the individual monograph.

This degree of precision is the minimum acceptable for determining that the final product complies with the official requirements and may be inadequate for those deciding, for example, the potency which should be stated on the label or used as the basis for calculating the quantity of an antibiotic to be incorporated in a preparation. In such circumstances, assays of greater precision may be desirable with, for instance, fiducial limits of error of the order of 98 per cent to 102 per cent. With this degree of precision, the lower fiducial limit lies close to the estimated potency. By using this limit, instead of the estimated potency, to assign a potency to the antibiotic either for labelling or for calculating the quantity to be included in a preparation, there is less likelihood of the final preparation subsequently failing to comply with the official requirements for potency.

2.2.11. Sterility

The test for sterility is applied to pharmacopoeial articles that are required according to the Pharmacopoeia to be sterile. However, a satisfactory result only indicates that no

contaminating viable microorganisms have been found in the sample examined in the conditions of the test. If the number of microorganisms present in a given amount of the article under examination is large, the probability of detecting them increases. Very low levels of contamination cannot be detected on the basis of random sampling of a lot. Moreover, if contamination is not uniform throughout the lot, random sampling cannot detect contamination with any certainty. Compliance with the test for sterility alone cannot therefore provide absolute assurance of freedom from microbial contamination. Greater assurance of sterility must come from reliable manufacturing procedures and compliance with good manufacturing practices.

The test must be carried out under aseptic conditions designed to avoid accidental contamination of the product during testing. For achieving these conditions, a grade A laminar air-flow cabinet or an isolator is recommended. The test environment has to be adapted to the way in which the tests are performed. Precautions taken for this purpose should not adversely affect any microorganisms, which are to be revealed in the tests. The working conditions in which the tests are carried out should be monitored regularly by appropriate sampling of the air and surfaces of the working area and by carrying out control tests.

The test is designed to reveal the presence of microorganisms in the samples used in the test; interpretation of the results of testing is based on the assumption that all units of an article or the entire bulk product or the contents of every container of the filled product in a lot or batch, had they been tested, would also have given the same results. Since all the units or the bulk or all the containers cannot be tested, a sufficient number of samples of units or of containers should be examined to give a suitable degree of confidence in the results of the tests.

No sampling plan for applying the tests to a specified proportion of discrete units selected from a batch is capable of demonstrating that all of the untested units are in fact sterile. Therefore, in determining the number of units to be tested, the manufacturer should have regard to the environmental conditions of manufacture, the volume of preparation per container and other special considerations particular to the preparation being examined. Table 1 gives guidance on the minimum number of containers recommended to be tested in relation to the number of items in the batch on the assumption that the preparation has been manufactured under conditions designed to exclude contamination.

If the contents of each container are of sufficient quantity, they may be divided so that equal appropriate portions are added to each of the specified media. If any container does not contain sufficient quantity of material to inoculate each of the specified media, use twice the minimum number of containers indicated in the Table 1.

Table 1

Number of containers in the batch*	Minimum number of containers recommended to be tested **
1. *Parenteral preparations*	
Not more than 100 containers	10 per cent or 4 containers, whichever is greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2 per cent or 20 containers, whichever is less
For large-volume parenterals	2 per cent or 10 containers, whichever is less, unless otherwise justified and authorised
2. *Ophthalmic and other non-parenteral preparations*	
Not more than 200 containers	5 per cent or 2 containers, whichever is greater
More than 200 containers	10 containers
3. *Surgical dressings and devices*	
Catgut, surgical sutures and other sterile medical devices for use	2 per cent or 5 packages, whichever is greater, up to a maximum of 20 packages
Not more than 100 packages	10 per cent or 4 packages, whichever is greater
More than 100 but not more than 500 packages	10 packages
More than 500 packages	2 per cent or 20 packages, whichever is less
4. *Bulk solids*	
Upto 4 containers	Each container
More than 4 containers but not more than 50 containers	20 per cent or 4 containers, whichever is greater
More than 50 containers	2 per cent or 10 containers, whichever is greater

* If the batch size is unknown use maximum number of articles prescribed

**If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

This sampling is, however, applicable at manufactures, not applicable to the inspectors under the Drugs and Cosmetics Act for drawing statutory samples and for government analyst.

Culture Media

Media for the tests may be prepared as described below, or equivalent commercially available dehydrated mixtures yielding similar formulations may be used provided that when reconstituted as directed by the manufacturer, they comply with the growth promotion test. Other media may be used provided that they have been shown to sustain the growth of a wide range of microorganisms.

The following culture media have been found to be suitable for the test. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soyabean-casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

Fluid Thioglycollate Medium – For use with clear fluid products.

L-Cystine	0.5 g
Sodium chloride	2.5 g
Dextrose monohydrate/anhydrous	5.5 g/5.0 g
Granular agar (moisture content less than 15 per cent, w/w)	0.75 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 ml
Resazurin sodium solution (0.1 per cent), freshly prepared	1.0 ml
Distilled water to	1000 ml
pH of the medium after sterilisation	7.1 ± 0.2

Mix the ingredients other than the thioglycollate or thioglycollic acid and the resazurin sodium solution, in the order given above, in a mortar, with thorough grinding. Stir in some heated *distilled water*, transfer to a suitable container, add the remainder of the *distilled water*, and complete the solution by heating in a boiling water-bath. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1M sodium hydroxide* so that, after sterilisation, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix and distribute the medium into suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilise in an autoclave at 121° for 20 minutes. If the medium is to be stored, cool promptly to 25° and store at 2° to 25°, avoiding excess of light. If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by reheating in a water-bath or in free-flowing steam until the pink colour disappears, and cooling rapidly, taking care to prevent the introduction of non-sterile air into the container. When ready for use, not more than the upper one-tenth of the medium should have a pink colour. Medium more than 4 weeks old should not be used.

Use fluid thioglycollate medium by incubating it at 30° to 35°. For the products containing a mercurial preservative that can not be tested by the membrane filtration method, fluid thioglycollate medium incubated at 20° to 25° may be used instead of soya-bean casein digest medium provided that it had been validated as described in growth promotion test.

Alternative Thioglycollate Medium — For use with turbid and viscid products and for devices having tubes with small lumina.

L-Cystine	0.5 g
Sodium chloride	2.5 g
Dextrose monohydrate/anhydrous	5.5 g/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 ml
Distilled water to	1000 ml
pH of the medium after sterilisation	7.1 ± 0.2

Heat the ingredients in a suitable container until solution is effected. Mix, add *1M sodium hydroxide*, if necessary, so that, after sterilisation, the medium will have a pH of 7.1 ± 0.2. Filter, if necessary, place in suitable vessels and sterilise at 121° for 20 minutes. Store at a temperature between 2° and 25° in a sterile sealed container, unless it is intended for immediate use.

The medium is freshly prepared or heated in a water-bath and allowed to cool just prior to use. It should not be reheated.

Use alternative thioglycollate medium in a manner that will assure anaerobic conditions for the duration of the incubation at 30° to 35°.

Soyabean-casein Digest Medium

Pancreatic digest of casein	17.0 g
Papaic digest of soyabean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.5 g
Dextrose monohydrate/anhydrous	2.5 g/2.3 g
Distilled water to	1000 ml
pH of the medium after sterilisation	7.3 ± 0.2

Dissolve the solids in *distilled water*, warming slightly to effect solution. Cool to room temperature and add, if necessary, sufficient *1M sodium hydroxide* so that after sterilisation the

medium will have a pH of 7.3 ± 0.2. Filter, if necessary, distribute into suitable containers and sterilise in an autoclave at 121° for 20 minutes.

Use soyabean-casein digest medium by incubating it at 20° to 25° under aerobic conditions.

Media for Penicillins and Cephalosporins

Where sterility test media are to be used in Method B described under Test Procedures modify the preparation of fluid thioglycollate medium and the soyabean-casein digest medium as follows. To the containers of each medium, transfer aseptically a quantity of penicillinase sufficient to inactivate the amount of antibiotic in the sample under test. Determine the appropriate quantity of penicillinase to be used for this purpose by using a penicillinase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power.

NOTE — Supplemented penicillinase media can also be used in Method A.

Alternatively (in an area completely separate from that used for sterility testing) confirm that the appropriate quantity of penicillinase is incorporated into the medium, following either method under Validation of Tests, using less than 100 CFU of *Staphylococcus aureus* (ATCC 6538) as the challenge. Typical microbial growth of the inoculated culture must be observed as a confirmation that the penicillinase concentration is appropriate.

Suitability of Media

The media used should comply with the following tests, carried out before or in parallel with the test on the preparation under examination.

Sterility. Incubate portions of the media for 14 days at the temperatures indicated under each medium. No growth of microorganisms occurs.

Growth Promotion Test. Test each autoclaved load of each lot of the medium for its growth-promoting qualities using suitable strains of microorganisms indicated in Table 2.

Inoculate duplicate portions of each medium with a small number (not more than 100 CFU) of the microorganisms specified, using separate portions of the medium for each of the microorganisms and incubating according to the conditions specified in Table 2.

The media are suitable if a clearly visible growth of the microorganisms occurs. The tests may be conducted simultaneously with any test for sterility done using the same lot of media. However, such tests will be considered invalid if the test media show inadequate growth response.

Table 2

Medium	Test micro-organism	Incubation		
		Temp (°)	Duration	Type of microorganism
Fluid Thioglycollate	1. *Clostridium sporogenes* (ATCC[1] 19404)	30 to 35	3 days	Anaerobic
	2. *Staphylococcus aureus* (ATCC 6538)	30 to 35	3 days	Aerobic
	3. *Pseudomonas aeruginosa* (ATCC 9027)[2]	30 to 35	3 days	Aerobic
Alternative Thioglycollate	1. *Bacteroides vulgatus* (ATCC 8482)[3]	30 to 35	3 days	Anaerobic
	2. *Clostridium sporogenes* (ATCC 19404)	30 to 35	3 days	Anaerobic
	3. *Bacillus subtilis* (ATCC 6633; NCIMB[4] 8054)	30 to 35	3 days	Aerobic
Soyabean-Casein Digest	1. *Aspergillus brasiliensis* (ATCC 16404)	20 to 25	5 days	Aerobic
	2. *Candida albicans* (ATCC 10231; ATCC 2091; NCYC[5] 854)	20 to 25	5 days	Aerobic
	3. *Bacillus subtilis* (ATCC 6633; NCIMB 8054)	20 to 25	3 days	Aerobic

1. Available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.

2. An alternative micro-organism is *Kocuria rhizophila* (ATCC No. 9341).

3. If a spore-forming organism is desired, use *Clostridium sporogenes* (ATCC No. 11437) at the incubation temperatures indicated in the Table.

4. Available from National Collection of Industrial and Marine Bacteria Ltd, 23 St Machar Drive, Aberdeen, AB2 1RY, Scotland.

5. Available from National Collection of Yeast Cultures, AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA, England.

NOTE — Seed lot culture maintenance techniques (seed-lot systems) should be used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

If freshly prepared media are not used within 2 days, they should be stored in the dark, preferably at 2° to 25°. Finished media, if stored in unsealed containers, may be used for not more than one month provided they are tested within one week of use.

Validation of Tests. Carry out a test as described under Test Procedures using exactly the same methods with the following modifications.

Membrane Filtration. After transferring the contents of the container or containers to be tested to the membrane add an inoculum of a small number of viable microorganisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

Direct Inoculation. After transferring the contents of the container or containers to be tested to the culture medium add an inoculum of a small number of viable microorganisms (not more than 100 CFU) to the medium.

In both cases use the same microorganisms as those described under Growth Promotion Test. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the inoculation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product under examination, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. A suitable sterile neutralising agent may be used where the preparation under examination has antimicrobial activity. If a neutralising agent is not available, modify the amounts of the preparation and medium to be used in order to eliminate antimicrobial activity and repeat the validation test. Where the specified amounts of the preparation have antimicrobial activity in the medium, increase the quantities of medium so that the specified quantity of the preparation is sufficiently diluted to prevent inhibition of growth.

This validation is performed

(a) when the test for sterility has to be carried out on a new product,

(b) whenever there is a change in the experimental conditions of the test.

The validation may be performed simultaneously with the test for sterility of the substance or preparation under examination.

Test Procedures

Either of the following methods, Method A – Membrane Filtration or Method B – Direct Inoculation, may be followed. Method A is to be preferred where the substance under examination is (a) an oil, (b) an ointment that can be put into solution, (c) a non-bacteriostatic solid not readily soluble in the culture medium, and (d) a soluble powder or a liquid that possesses bacteriostatic and/or fungistatic properties. Appropriate negative controls are included.

For liquid products where the volume in a container is 100 ml or more, Method A should be used.

Select the number of samples to be tested from Table 1 and use them for the culture medium for bacteria and the culture medium for fungi.

General. The exterior surface of ampoules and closures of vials and bottles should be cleaned with a suitable antimicrobial agent and access to the contents should be gained in a suitable aseptic manner. If the contents are packed in a container under vacuum, sterile air should be admitted by means of a suitable sterile device, such as a needle attached to a syringe barrel filled with non-absorbent cotton.

Method A – Membrane Filtration

The method calls for the routine use of positive and negative controls. A positive control is small number (not more than 100 CFU) of microorganisms specified in separate portion of each medium.

Apparatus

A suitable unit consists of a closed reservoir and a receptacle between which a properly supported membrane of appropriate porosity is placed. A membrane generally suitable for sterility testing has a nominal pore size not greater than 0.45 µm and diameter of approximately 50 mm, the effectiveness of which in retaining microorganisms has been established. Cellulose nitrate filters are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for strongly alcoholic solutions. Preferably assemble and sterilise the entire unit with the membrane in place prior to use. Where the sample to be tested is an oil, sterilise the membrane separately and, after thorough drying, assemble the unit using aseptic precautions. Alternatively, an apparatus can be designed so that the solution to be examined can be introduced and filtered under aseptic conditions. It permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Diluting Fluids

Fluid A. Dissolve 1 g of peptic digest of animal tissue (such as bacteriological peptone) or its equivalent in *water* to make 1

litre, filter or centrifuge to clarify, adjust to pH 7.1 ± 0.2, dispense into flasks in 100 ml quantities and sterilise at 121° for 20 minutes.

NOTE — Where fluid A is to be used in performing the test for sterility on a specimen of the penicillin or cephalosporin class of antibiotics, aseptically add a quantity of sterile penicillinase to the fluid A to be used to rinse the membrane(s) sufficient to inactivate any residual antibiotic activity on the membrane(s) after the solution of the specimen has been filtered.

Fluid B. If the test sample contains lecithin or oil, use fluid A to each litre of which has been added 1 ml of *polysorbate 80*, adjust to pH 7.1 ± 0.2, dispense into flasks and sterilise at 121° for 20 minutes.

NOTE — A sterile fluid shall not have antibacterial or antifungal properties if it is to be considered suitable for dissolving, diluting or rinsing a preparation being examined for sterility.

Quantities of Sample to be used

For parenteral preparations. Whenever possible use the whole contents of the container, but in any case not less than the quantities prescribed in Table 3, diluting where necessary to about 100 ml with a suitable diluent such as fluid A.

For ophthalmic and other non-parenteral preparations. Take an amount within the range prescribed in column (A) of Table 4, if necessary, using the contents of more than one container, and mix thoroughly. For each medium use the amount specified in column (B) of Table 4, taken from the mixed sample.

Method of Test

For aqueous solutions. Prepare each membrane by aseptically transferring a small quantity (sufficient to moisten the membrane) of fluid A on to the membrane and filter it. For each medium to be used, transfer aseptically into two separate membrane filter funnels or to separate sterile pooling vessels prior to transfer not less than the quantity of the preparation under examination that is prescribed in Table 3 or Table 4. Alternatively, transfer aseptically the combined quantities of the preparation under examination prescribed in the two media onto one membrane. Draw the liquid rapidly through the filter with the aid of vacuum. If the solution under examination has antimicrobial properties, wash the membrane(s) by filtering through it (them) not less than three successive quantities, each of 100 ml, of sterile fluid A. Do not exceed a washing cycle of 5 times 100 ml per filter, even if it has been demonstrated during validation that such a cycle does not fully eliminate the antimicrobial activity. The quantities of fluid used should be sufficient to allow growth of a small inoculum of organisms (approximately 100 CFU) sensitive to the antimicrobial substance in the presence of the residual inhibitory material on the membrane.

After filtration, aseptically remove the membrane from the holder, transfer the whole membrane or cut it aseptically into 2 equal parts. Transfer one half to each of two suitable media. Use the same volume of each medium as in the procedure for validation of Tests. Alternatively, transfer the medium on to the membrane in the apparatus. Incubate the media for not less than 14 days.

Observe the containers of media periodically during the 14 days of incubation. If the test specimen is positive before 14 days of incubation, further incubation is not necessary.

For liquids immiscible with aqueous vehicles, and suspensions. Carry out the test described under For aqueous solutions but add a sufficient quantity of fluid A to the pooled sample to achieve rapid filtration. Sterile enzyme preparations such as penicillinase or cellulase may be added to fluid A to aid in dissolving insoluble substances. If the substance being examined contains lecithin, use fluid B for diluting.

For oils and oily solutions. Filter oils or oily solutions of sufficiently low viscosity without dilution through a dry membrane. Dilute viscous oils as necessary with a suitable sterile diluent such as *isopropyl myristate* that has been shown not to have antimicrobial properties under the conditions of the test. Allow the oil to penetrate the membrane and filter by applying pressure or by suction, gradually. Wash the membrane by filtering through it at least three successive quantities, each of approximately 100 ml, of sterile fluid B or any other suitable sterile diluent. Complete the test described under

For aqueous solutions, beginning at the words "After filtration,……".

For ointments and creams. Dilute ointments in a fatty base and emulsions of the water-in-oil type to give a fluid concentration of 1 per cent w/v, by heating, if necessary, to not more than 40° with a suitable sterile diluent such as *isopropyl myristate* previously rendered sterile by filtration through a 0.22 µm membrane filter that has been shown not to have antimicrobial properties under the conditions of the test. Filter as rapidly as possible and complete the test as described under For oils and oily solutions, beginning at the words "Wash the membrane by……". In exceptional cases, it may be necessary to heat the substance to not more than 44° and to use warm solutions for washing the membrane.

NOTE — For ointments and oils that are insoluble in isopropyl myristate, *use Method B.*

For soluble solids. For each medium, dissolve not less than the quantity of the substance under examination, as prescribed in Tables 3 and 4, in a suitable sterile solvent such as fluid A and carry out the test described under For aqueous solutions using a membrane appropriate to the chosen solvents.

Table 3

Quantity in each container of injectable preparation	Minimum quantity to be used for each culture medium
For liquids	
Less than 1 ml	Total contents of a container
1 - 40 ml	Half the contents of a container, but not less than 1 ml
More than 40 ml but not more than 100 ml	20 ml
More than 100 ml	10 per cent of the contents of a container but not less than 20 ml
Antibiotic liquids	1 ml
Other preparations soluble in water or in isopropyl myristate	The whole contents of each container to provide not less than 200 mg
Insoluble preparations, creams and ointments to be suspended or emulsified	The whole contents of each container to provide not less than 200 mg
For solids	
Less than 50 mg	Total contents of a container
50 mg or more but less than 300 mg	Half the contents of a container, but not less than 50 mg
300 mg to 5 g	150 mg
More than 5 g	500 mg
For catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30 cm long)
For surgical dressings/cotton/gauze (in packages)	100 mg per package
For sutures and other individually packed single use materials	The whole device
Other medical devices	The whole device or material, cut into pieces or disassembled

Table 4

Type of preparation	Quantity to be mixed (A)	Quantity to be used for each culture medium (B)
Ophthalmic solutions; other than non-parenteral liquid preparations	10 to 100 ml	5 to 10 ml
Other preparations; preparations soluble in water or appropriate solvents; insoluble preparations to be suspended or emulsified (ointments and creams)	1 to 10 g	0.5 to 1 g
Absorbent cotton		Not less than 1 g*

*One portion.

For solids for injection other than antibiotics. Constitute the test articles as directed on the label, and carry out the test as described under For aqueous solutions or For oils and oily solutions, as applicable.

NOTE — If necessary, excess diluent may be added to aid in the constitution and filtration of the constituted article.

For antibiotic solids, bulks, and blends. Aseptically remove a sufficient quantity of solids from the appropriate amount of containers prescribed in Table 3, mix to obtain a composite sample, equivalent to about 6 g of solids, and transfer to a sterile flask. Dissolve in about 200 ml of fluid A, and mix. Carry out the test as described under For aqueous solutions.

For antibiotics in packages of 5 g or less. From each of 20 containers, aseptically transfer about 300 mg of solids into a sterile flask, dissolve in about 200 ml of fluid A and mix, or constitute as directed on the label of containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids into a sterile flask, dissolve in about 200 ml of fluid A, and mix. Carry out the test as described under For aqueous solutions or For oils and oily solutions, as appropriate.

Devices with pathways labelled sterile. Aseptically pass not less than 10 pathway volumes of fluid B through each of not less than 20 devices tested. Collect the fluid in sterile containers and filter the entire volume through the membrane filter the entire volume through the membrane filter funnel(s) as described under For aqueous solution or oil or oily solution.

In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test and express the contents into a sterile polling vessel. Proceed as directed above.

For catheters where the inside lumen and outside surface are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or full the lumen with medium and then immerse the intact unit.

Method B – Direct Inoculation

Quantities of Sample to be used

The quantity of the substance or preparation under examination to be used for inoculation in the culture media varies according to the quantity in each container. Follow the directions given in Table 3.

Method of Test

For aqueous solutions and suspensions. Remove the liquid from the test containers with a sterile pipette or with a sterile syringe or a needle. Transfer the quantity of the preparation under examination prescribed in Table 4 directly into the culture medium so that the volume of the preparation under examination is not more than 10 per cent of the volume of the medium, unless otherwise prescribed. When the quantity in a single container is insufficient to carry out the tests, the combined contents of two or more containers are to be used to inoculate the media.

If the preparation under examination has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

Incubate the inoculated media for not less than 14 days (irrespective of method of sterilization). Observe the containers of media periodically during the 14 days of incubation. If the test specimen is positive before 14 days of incubation, further incubation is not necessary.

For oils and oily solutions. Use media to which has been added a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example, *polysorbate 80* at a concentration of 10 g per l and which has been shown not to have any antimicrobial properties under the conditions of the test. Carry out the test as described under for aqueous solutions and suspensions.

During the incubation period shake the cultures gently each day. However, when thioglycollate medium or other similar medium is used for the detection of anaerobic microorganisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

For ointments and creams. Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as fluid A. Transfer the diluted product to a medium not containing an emulsifying agent. (Before use, test the emulsifying agent to ascertain that in the concentration used it has no significant antimicrobial effects during the time interval for all transfers). Mix 10 ml of the fluid mixture so obtained with 80 ml of the medium and proceed as directed under For aqueous solutions and suspensions.

For solids. Transfer the quantity of the preparation under examination to the quantity of medium specified in Table 4 and mix. Proceed as directed under For aqueous solutions and suspensions.

For surgical dressings and related articles. From each package under examination, aseptically remove two or more portions of 100 to 500 mg each from the innermost part of the sample. From individually packaged, single-use materials,

aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed under For aqueous solutions and suspensions.

For sterile devices. For articles of such size and shape that permit complete immersion in not more than 1000 ml of the culture medium, for large device , immerse completely those portion of the device that comes into direct contact with patient test the article, using the appropriate media, and proceed as directed under For aqueous solutions and suspensions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces or fill the lumen with medium, and then immerse the intact unit.

Observation and Interpretation of Results

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be easily determined by visual examination, 14 days after the beginning of incubation, transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the preparation under examination complies with the test for sterility. If evidence of microbial growth is found, the preparation under examination does not comply with the test for sterility. Do not repeat the test unless it can be clearly shown that the test was invalid for causes unrelated to the preparation under examination. The test may be considered invalid only when one or more of the following conditions are fulfilled:

(a) microbial growth is found in the negative controls;

(b) data on microbial monitoring of the sterility testing facility show a fault;

(c) a review of the testing procedure used for the test in question reveals a fault;

(d) after identifying the microorganisms isolated from the containers showing microbial growth, the growth may be ascribed without any doubt to faults with respect to the materials and/or technique used in conducting the test procedure.

If the test is declared to be invalid, repeat with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the preparation under examination complies with the test for sterility. If microbial growth is found in the repeat test and confirmed microscopically, the preparation under examination does not comply with the test for sterility.

2.2.12. Thiomersal

Standard Preparation. Weigh accurately about 0.1 g of *thiomersal*, previously dried over *phosphorus pentoxide* at a pressure not exceeding 2 kPa for 24 hours, and dissolve in sufficient *water* to produce 1000 ml. Prepare just before use dilutions of this solution with *water* to produce standard solutions containing in each ml 5.0 µg (low level) and 10.0 µg (high level) of thiomersal.

Test medium

Pancreatic digest of casein	10.0 g
Beef extract	3.0 g
Yeast extract	1.5 g
Agar	15.0 g
Sodium chloride	3.0 g
Sucrose	1.0 g
Water to	1000 ml

Dissolve with the aid of heat, adjust the pH to 7.4 to 7.6 and sterilise by maintaining at 121° for 20 minutes.

Test organism

The test organism recommended for this assay is *Micrococcus luteus* (ATCC 10240; NCIB 8994).

Preparation of Inoculum and Assay Plates

Maintain the test organism by regular sub-culturing on slopes of the test medium. After incubation at 30° for 18 to 24 hours, emulsify the growth in about 10 ml of sterile *normal saline*.

To 150 ml of the test medium kept at a temperature of 46° to 48° add 1.5 ml of the well-shaken inoculum, swirl gently and mix well. Immediately pour the inoculated medium into Petri dishes or large rectangular plates to give a depth of 3 to 4 mm. Cool and transfer to a cold place until the medium is set. Prior to use, prepare cavities approximately 8 mm in diameter in the solid medium.

Procedure

From an accurately measured volume of the preparation under examination prepare sample solutions containing two levels, namely about 5 µg and 10 µg of thiomersal and carry out method A, Cylinder-plate or Cup-plate Method, two-level factorial assay described under microbiological assay of antibiotics (2.2.10), incubating the plates at 30° for about 18 hours.

Calculate the result by standard statistical methods. The fiducial limits of error are not less than 90 per cent and not more than 100 per cent of the estimated value.

If the value obtained for the samples is lower than 50 per cent or greater than 150 per cent of the standard, the determination is invalid and should be repeated using higher or lower dilutions of the sample solution.

2.2.13. Urinary Excretion of Dextrans

Place two rabbits in separate metabolism cages and collect the urine of each for 48 hours. Into the marginal ear vein of each rabbit inject over a period of 5 to 10 minutes a dose of 20 ml per kg of body weight of the preparation under examination and collect the urine excreted in the first 48 hour after injection. All the urine should be collected in clean glass vessels under *toluene*. Measure the total volume excreted by each of the rabbits and filter the urine.

Dialyse through a suitable membrane 10 ml of each sample of urine against running water for 24 hours and against *water* for further 24 hours. Transfer to a 25-ml volumetric flask by means of a Pasteur pipette, rinse the membrane with 6 to 8 ml of *water* and dilute the combined urine and rinsings to 25 ml with *water*. To 3.0 ml of this solution, or a suitable volume diluted to 3.0 ml with *water* in a test-tube cooled in *water*, add carefully 6.0 ml of a 0.2 per cent w/v solution of *anthrone* in a mixture of 19 volumes of *sulphuric acid* and 1 volume of *water* so that the solutions form layers. Mix the contents of the tube immediately, place in a water-bath and heat for 5 minutes with precautions against loss of water by evaporation. Cool and compare the colour of the solution with that of the blank prepared in the same manner from 3.0 ml of *water* using a colorimeter fitted with a red filter. Determine the apparent amount of dextrose in the solution from a reference curve prepared by treating 3.0-ml quantities of solutions of *anhydrous dextrose* by the same process (suitable amounts of dextrose for this purpose are 15, 30, 60 and 90 µg per 3.0 ml).

Calculate the percentage of dextrans for each rabbit from the expression

$$\frac{90(x - y)}{w}$$

where, x = the total apparent amount of dextrose, in mg excreted in the first 48 hours after injection,

y = the total apparent amounts of dextrose, in mg excreted in the 48 hours before injection,

w = weight, in mg, of dextrans injected.

The result of the test is determined on the mean of the result for the two rabbits and must not exceed 30 per cent.

2.2.14. Immunochemical Methods

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen antibody complex may be detected, and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of the immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardize the components of the immunoassay and to use, wherever available international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is, a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions, it is important to ascertain that the kits are suitable for the analysis of the substance under examination, with particular reference to selectivity and sensitivity. Guidance concerning immuno-assay kits is provided by the World Health Organisation, Technical Series 658 (1981).

Methods in which a labelled antigen or a labelled antibody is used

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radio-immunoassay". All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

Methods in which an unlabelled antigen or antibody is used

Immunoprecipitation methods

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants which gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase in sensitivity can be obtained by using antigen or antibody-coated particles (e.g. latex) as reactants.

In flocculation methods, stepwise dilutions of one of the reactants is usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are prescribed in tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as simple; when it involves related but not serologically identical reactants, the system is complex and where several serologically unrelated reactants are involved, the system is multiple.

In simple diffusion methods, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

Single radial immunodiffusion (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactant has been established, the circular precipitation area originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In double diffusion methods, concentration gradients are established for both reactants. Both antigen and antibody diffuse from separate sites into an initially immunologically neutral gel.

Comparative double diffusion methods are used for qualitatively comparing various antigens versus a suitable antibody or vice versa. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens/antibodies can be distinguished.

Immunoelectrophoretic methods

Immunoelectrophoresis (IE) is a qualitative technique combining two methods: Gel electrophoresis followed by immunodiffusion.

Crossed immunoelectrophoresis is a modification of the IE method. It is suitable both for qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions under determination, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out perpendicular to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

Electroimmunoassay, often referred to as rocket immunoelectrophoresis is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or vice versa. The electrophoresis of the antigen under determination is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

Counter-immunoelectrophoresis is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an electric field depending on the different charges. Dilutions of a standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualisation and characterization of immunoprecipitation lines

These may be performed by selective or non-selective strains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterization of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

Validation of the method

Validation criteria

A quantitative immunochemical method is not valid unless:

1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound.
2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity.
3) The limit of quantitation is below the acceptance criteria stated in the individual monograph.
4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs.

5) The order to which the assay is performed does not give rise to systematic errors.

Validation methods

In order to verify these criteria, the validation design includes the following elements:

1) The assay is performed at least in triplicate.
2) The assay includes at least 3 different dilutions of the standard preparation and 3 different dilutions of sample preparations of presumed activity similar to the standard preparation.
3) The assay layout is randomized.
4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared.
5) The test includes the measurement of non-specific binding of the labelled reactant.
6) For displacement immunoassay:
   a) maximum binding (zero displacement) is determined;
   b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

Statistical calculation

To analyse the result, response curves for test and standard may be analysed by the methods stated under Statistical Analysis of Results (5.7).

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the value for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

2.2.15. Host Cell Proteins and Host Cell DNA

Host Cell Proteins (HCP)

HCP are cell based impurities that originate from the biological systems used for the production of the therapeutic proteins. The level of HCP in the cell culture fluid collected for harvest is primarily a function of the expression system, culture conditions, and other downstream unit operations that are used for recovery of the product of interest. Evidence gathered over the years has demonstrated that the HCP can pose risk towards product degradation (via residual proteases) or at worse can influence immunogenicity of the product. Thus, it is necessary to define acceptable levels of these impurities to ensure safe and efficacious product.

An array of multiple orthogonal analytical techniques can be used for HCP detection and quantitation. The technique used should be able to detect HCP across a wide dynamic range and be able to trace the changing HCP population through each step of the bioprocess. Immunoassay techniques (ELISA-Enzyme Linked Immunosorbent Assay) based on polyclonal antibodies raised to the host cell proteins of the biological system used to synthesize particular therapeutic protein have been widely used. This technique provides the total amount of all the HCP present in the tested sample. However, the sensitivity of the assay depends on the antibody serum used which dictates the coverage of the HCP spectrum. The technique also does not allow toundertake a risk based assessment of HCP as per the Quality by Design (QbD) paradigm of process and product development. Therefore, additional/orthogonal techniques to complement these ELISA methods should clearly be adopted. Gel based techniques (1D-/2D-gel electrophoresis) are generally used during process development. Additionally, 2D-DIGE is used to gain information about the change in HCP level across the various stages of the process as the technique allows analysis of multiple samples on the same gel. Western blot analysis of 1D-/2D-gels is also undertaken to detect those HCP that are missed with above techniques or in those cases where low abundance HCP are masked by relative enormity of target protein. However, the concerns of sensitivity and coverage still remains as western blotting also depends on the pool of anti-HCP antibodies used.

Mass spectrometry based methods in conjunction with various separation techniques such as liquid chromatography / capillary electrophoresis has emerged as major orthogonal technique to ELISA for HCP analysis. The technique has been employed not only to catalogue individual HCP present throughout entire bioprocess train but also to accurately quantify these diverse HCP species that vary from each other in terms of complexity, risk posed and abundance using wider proteomic analytical methods such as iTRAQ labelling and several advanced targeted MS techniques such as multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM).

Based on the above mentioned considerations, to accurately detect and measure individual HCP, a target of 100 ng/mg of HCP in the final drug product has been set as an acceptable limit. However, a case-by-case evaluation is deemed necessary to mitigate any risk with respect to safety and/or efficacy of a therapeutic product in view of the process capability to remove these impurities. In cases where appropriate clearance studies have been rigorously performed and adequately demonstrated as a part of the process validation, the need for HCP analysis has been relaxed.

In conclusion, the method for HCP monitoring and measurement should be scientifically sound and should encompass following:

1. The assay should be able to measure HCP across a wide dynamic range (from very low to very high concentrations of individual HCP).
2. The assay should have high sensitivity (ability to measure low concentration HCP in presence of high concentration of target protein).
3. The technique should provide sufficient information for establishing the safety and efficacy of the product. The analytical tools elucidated in this chapter (mass spectrometry based methods, gel based techniques, western blotting etc.,) should be used as deemed necessary for achieving this objective.
4. Wherever provided, the manufacturer should comply with the specifications prescribed in the individual monographs.

Host Cell DNA (HCD)

Like HCP, host cell DNA are also host cell derived molecules present as impurity in the drug substance (biotherapeutic). These impurities generally get co-purified with the drug substance during product purification. Some of these impurities are known to cause toxicity. Therefore, it is necessary to remove these impurities to an acceptable level to mitigate any adverse events. Guideline to this effect varies across regulatory bodies globally. However, a wider consensus, evident from similar specification limit recommended by WHO and FDA, has been at 100 pg per therapeutic dose or 10 ng per dose for those therapeutics that are administered at higher doses (e.g., mAbs).

A number of techniques are at disposal for HCD analysis. These include PicoGreen analysis, hybridization techniques, qPCR, and the Threshold assay. HCD analysis should be carried out not only on the final drug product but also in the process intermediates. Lot-to-lot values of the HCD in the drug product should adhere to the established lot release limits. It is to be noted that the requirement of HCD testing may be relaxed if the removal of DNA burden is appropriately demonstrated using rigorous clearance study carried out as a part of process validation.

Quantitive Polymerase Chain Reaction (qPCR)

qPCR is a sequence specific assay that is an extension of polymerase chain reaction (PCR) and is among the most widely used technique for quantitation of nucleic acids (HCD). The technique exploits the ability to monitor the amplification of DNA with a PCR in real time to determine the quantity of target in the reaction. Data are collected throughout the process to monitor the increase in PCR product formation, enabling quantitative determination of the starting amounts of DNA in a sample. Different chemistries can be used to detect HCD using qPCR. It includes SYBR Green I dye, High resolution melting dyes (HRM dyes) and sequence-specific reporters such as hybridization and 5′-nuclease probes.

The key steps in performing a qPCR analysis involves following steps:

1. Denaturation of DNA: DNA in the test sample is denatured to single stranded DNA (ssDNA).
2. Fluorescent probes are hybridized to their respective complementary sequences on the single stranded DNA.
3. Exponential amplification of target DNA occurs and the fluorescence released during this step is directly proportional to the amplified DNA.

Filter-based hybridization analysis

This technique involves binding of DNA probes to denatured and immobilized host-cell DNA. Probes are labeled with radioactive tags or fluorescent dyes and bound to complementary targets during hybridization. Signal detection is achieved with autoradiography or by phosphor- or fluorescence-imaging systems, and the signal detected is proportional to the amount of DNA immobilized on a filter. Depending on the probe used, this assay can be either specific or non-sequence specific.

DNA in the test sample is denatured to give single-stranded DNA, immobilized on a nitrocellulose or other suitable filter and hybridized with labelled DNA prepared from the host-vector manufacturing system (DNA probes). Although a wide variety of experimental approaches are available, hybridization methods for measurement of host-vector DNA meet the following criteria:

— DNA probes. Vero cells should be grown under the same conditions as those used in the vaccine manufacturing process and chromosomal DNA should be purified and used as probes. The purified DNA should be digested with appropriate restriction enzyme (for example, EcoRI, BamHI, etc.), deproteinized and purified. The concentration of DNA should be measured spectroscopically and the DNA should be stored at appropriate storage condition. Such DNA preparations should be labelled by radioactive or non-radioactive methods and used as probes in dot blot or slot blot analysis. The labelling technique should generate high specific activity probes so that 10 picograms of DNA can be detected when used in dot blot/slot blot experiments.

— Standardization and hybridization conditions. Each batch of Vero cell DNA prepared as described above should be calibrated and used as standards. Different amounts of denatured Vero cell DNA (0, 10, 25, 50, 100, 250, 500, 750 and 1000 picograms) should be loaded on a nitrocellulose/

nylon filter along with DNA purified from test samples (preferable in duplicate). Filters should be hybridized with labelled vero cell DNA probes. The stringency of hybridization conditions should be such as to ensure specific hybridization between the probes and standard DNA preparations.

— Calibration and standardization. Quantitative data are obtained by comparison with responses obtained using standard preparations. Chromosomal DNA probes and vector DNA probes are used with chromosomal DNA and vector DNA standards, respectively. Standard preparations are calibrated by spectroscopic measurements and stored in a state suitable for use over an extended period of time.

— Hybridization conditions. The stringency of hybridization conditions is such as to ensure specific hybridization between probes and standard DNA preparations and the drug substance must not interfere with hybridization at the concentrations used.

Sequence-independent techniques

These methods of HCD analysis do not require sequence template of the target DNA for its quantitation. Typical assay in this category includes PicoGreen assay and Threshold assay. While the former measures total double stranded DNA (dsDNA) in the sample using principles of fluorometry, latter employs a capture technique whereby a biotinylated single-stranded binding (SSB) protein and an anti-ssDNA antibody conjugated to urease bind simultaneously to the single stranded DNA (ssDNA) present in a sample.

These procedures does not require the use of specific heat or vector DNA as an assay standard. However, the method used must be validated to ensure parallelism with the DNA standard used, linearity of response and non-interference of either the drug substance or excipients of the formulation at the dilutions used in the assay. In addition, any other method or technique shall be validated and approved by the national regulatory authority.

The manufacturer should comply with the limit as prescribed in individual monographs.

2.2.16. Limes flocculation (Lf)

The Limes flocculation (Lf) of diphtheria toxin, diphtheria toxoid or diphtheria vaccine or tetanus vaccine is determined by incubating together the preparation under examination and the Standard preparation of diphtheria antitoxin or tetnus antitoxin for flocculation test in appropriate concentrations. When the concentration of toxin, or toxoid, is kept constant and the concentration of the antitoxin varied in mixtures of constant volume, the mixture flocculating first is that which contains the most nearly equivalent quantities of toxin, or toxoid and antitoxin.

Reference preparation

The Reference preparation is freeze-dried purified hyperimmune horse serum of the relevant antitoxin or other suitable preparation the activity of which has been determined in relation to the International reference preparation.

Suggested method

Carry out preliminary tests to determine the range of concentrations to be used. To each of a series of small tubes containing equal volumes of graded concentrations of the Reference preparation add a constant volume of a solution of the preparation under examination. In successive tubes the antitoxin concentration should increase by amounts not greater than one-tenth of the concentration in the middle of the range of concentrations. Choose a range in such a manner that the optimum flocculating mixture will occur in the middle of the range. Heat the tubes in a water-bath at 45° to 50° with half the fluid column immersed to obtain convection currents. Continue heating and observe the tubes until the most rapidly flocculating mixture has been determined. The number of Lf in the quantity of the preparation under examination is numerically equal to the number of Lf equivalents of antitoxin in this mixture. The error of a single determination has been estimated to be not more than 5.0 per cent.

2.2.17. DNA-Based Authentication Techniques

DNA-based authentication comprises various techniques which are used for analyzing DNA sequences and fingerprints. These techniques are employed in various fields such as taxonomy, physiology, embryology and genetics and have been successfully applied in the field of commercially important plants such as food crops and horticultural plants. The application of molecular methods to the authentication of Indian medicinal materials was initiated in the middle of 1990s when DNA-based techniques were used for differentiation of accessions collected at different geographic regions, differentiation of inter and intra-species variations, authentication of plants frequently substituted with species which are indistinguishable by macroscopic, microscopic or phytochemical methods, and for detection of adulterations. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological condition and environmental factors. They are not tissue-specific and can be detected at any stage of organism development. Only small sample amounts are needed for most methods. This makes DNA-based methods interesting for authentication of fragile, animal-derived and precious drugs. Despite these advantages, DNA-related techniques are not yet routinely used for

authentication of medicinal plants/herbs as the methods are relatively expensive and as their application can be problematic in case of dried or processed materials.

Hybridisation-based methods

Hybridisation-based methods use cloned DNA elements or synthetic oligonucleotides as probes to hybridise DNA. The probes are labeled with radioisotopes or with conjugated enzymes which catalyse a colour reaction to detect hybridization. DNA is either cleaved with restriction enzymes or first amplified by polymerase chain reaction (PCR), subsequently separated by gel electrophoresis and transferred to a solid support matrix.

Restriction fragment length polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. This method is mostly used in paternity cases or criminal cases to determine the source of a DNA sample, but also widely applied in genome mapping, marker-aided breeding and phylogenetic studies. In this technique, restriction polymorphisms are detected by using a hybridization probe. The quality of the assay is highly dependent on the choice of the DNA probe. Two types of DNA probes can be used, namely tandem repeats which occur as clusters among chromosomes and dispersed repeats which are scattered all over the chromosomes. Shorter repeating sequences (generally fewer than six base pairs) which are repeated from a few to many thousands of times, are abundant in the eukaryote genome. They are called microsatellites or single sequence repeat (SSR).

The major limitation of this method is its low sensitivity. It requires a large amount of high-quality genomic DNA, which is very difficult to obtain from processed herbal drugs. Also the stability and reproducibility are rather low because the quality of the assay highly depends on DNA quality and technical factors.

For Indian medicinal materials, PCR-RFLP is more suitable, as it requires a much lower amount of DNA. In this method a defined DNA sequence is first amplified by PCR and then selected restriction enzymes are employed to generate fragments unique to the respective species.

Polymerase chain reaction-based methods

PCR enzymatically multiplies particular DNA sequences or loci of a template DNA with the help of arbitrary or specific oligonucleotide primers.

It is not easy to standardize PCR-based authentication procedures for medicinal plants. Reasons for variations are different DNA polymerases used, different buffer formulations as well as different equipment used. Variations can be reduced if the same commercial kits or polymerase mixtures are used in each lab performing the same test. Problems also occur when the plant material of interest contains compounds such as phenolics which can interact with the DNA and directly inhibit DNA polymerase or damage the structural integrity of DNA. The way a plant is harvested, stored and processed can also influence DNA quality.

Various PCR-based techniques have been employed to authenticate and characterize medicinal plants, some of them do not require prior knowledge of the DNA sequence, for example Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Arbitrarily Primed PCR (AP-PCR) and Inter Simple Sequence Repeat (ISSR); while for others, such as Simple Sequence Repeat (SSR), prior sequence information is required.

Amplified fragment length polymorphism

This fingerprinting technique is based on the detection of multiple DNA restriction fragments by means of PCR amplification and has the capacity to detect thousands of independent loci. Genomic DNA is digested by appropriate restriction enzymes (preferably a hexa-cutter or tetra-cutter), which cut DNA at defined sequence sites. A subset of the resultant fragments is then ligated to synthetic double-stranded adaptors (DNA segments) at each end and subsequently amplified using two specific adaptor-homologous primers. The amplified and labeled restriction fragments are separated on denaturating gels or by capillary electrophoresis. The complexity of the AFLP profiles depends on the primers and restriction enzymes chosen and on the level of sequence polymorphism between the tested DNA samples.

The number of amplified bands of the preselected PCR is usually so high that a second round of PCR (selective PCR with fluorescent dyes) has to be performed to reduce the number of amplified products. This is done by using primers that possess 1-3 additional bases at the 3' end. Several combinations have to be tested for optimization. Digestion, ligation and two rounds of PCR make this method expensive and time-consuming. However, it is a very reliable and robust technology, and it has been widely applied in screening DNA markers linked to genetic traits, parentage analysis, forensic genotyping and population genetics However, the feasibility for the authentication of Indian herbs might be limited, because high-quality DNA, especially high-molecular-weight DNA, is required, which might be a problem in the case of processed or dried medicinal materials.

Arbitrarily primed PCR and random amplified polymorphic DNA

These methods have been simultaneously developed by two groups in 1990. Basically, instead of using region-specific PCR primers or adaptors, one single, arbitrarily chosen

oligonucleotide is used as both the forward and reverse primer in a PCR reaction. This sequence consists of about 10 nucleotides in case of RAPD and about 20 nucleotides in case of AP-PCR. A product is produced when the primer binds on opposite strands, in the reverse orientation and within an amplifiable distance. PCR fragments are generated from different locations of the genome, because there are multiple sites within the genome for the primer to bind. Thus, multiple loci may be examined simultaneously. Using series of different primers allows the generation of a genetic fingerprint of a plant or a specific species. RAPD analysis is a quick, rather easy and relatively cheap method; a major drawback is that reproducibility between laboratories has been shown to be low because the procedure is very sensitive to different PCR parameters, especially to slight variations in annealing temperature because of the low annealing temperatures required. Nevertheless, RAPD and AP-PCR have extensively been used to study the genetic diversity of various Indian medicinal plants, to discriminate different plant population and to differentiate plant batches of different provinces.

Inter simple sequence repeat (ISSR)

ISSR makes use of anchored primers to amplify simple sequence repeats. The primers are not totally arbitrary but are composed of repeated di-3, tri-3, tetra- or pentanucleotid motifs which are scattered throughout the eukaryote genome. As in RAPD analysis, a product is achieved when the same repeat is present in the two DNA strands within an amplifiable distance. Specificity and reproducibility can be enhanced by addition of a different base at 3' or 5'. Because longer primers are used, the method is more reliable and less sensitive to annealing temperature than RAPD analysis; however, the annealing temperature has to be optimized for each primer and species DNA. Compared with AFLP and SSR analysis, the method is rather quick, straightforward and economic.

Simple sequence repeat (SSR)

For this technique, prior sequence information is required. SSR markers or microsatellites (also termed simple sequence length polymorphism (SSLP) or sequence-tagged microsatellite site (STMD) are tandem repeats scattered throughout the genome. They can be amplified using primers that flank these regions. The technique has been successfully used to construct detailed generic maps of several organisms and to study genetic variation within populations of the same species as the markers are usually species-specific, their development is rather costly, but once they have been developed the method becomes quite inexpensive.

Sequencing-based markers and microarray techniques

Concerning these techniques, DNA sequences from the nuclear and chloroplast genome are used for identification of plants at several taxonomic levels. Certain sequences such as the 5S rDNA spacer and the internal transcribed spacer (ITS) regions between the 16S and 26S rDNA are highly variable and can thus be used for authentication at species level. Sequences such as nuclear 18S or plastidal rbcL, ndhF or matK however, are conserved among species and therefore suitable for discrimination at genus or family level. DNA sequence-based techniques have widely been used for authentication of Indian herbs.

In summary, DNA-based authentication methods have several advantages which make them suitable for the identification of Indian herbs:

- the techniques are independent from the physical form of the material;
- only a low amount of material is required;
- DNA-based markers represent the most basic signature of a living being and are therefore much less affected by environmental influences than phenotypic markers.

On the other hand, some limitations have to be considered:

- Some of the methods are strongly dependent on DNA quality, which might be a problem in the case of dried or processed materials;
- Certain plant compounds or fungal contamination may also influence DNA extraction or PCR reaction.

To establish a marker for identification of a particular species, DNA analysis of closely related species and/or varieties as well as common contaminants and adulterants is necessary, a very expensive and time consuming process.

The DNA-based investigation of herbal samples for unknown contaminants is at present extremely difficult and costly because of the broad spectrum of possible admixtures. More comprehensive databases of DNA fingerprints and DNA sequences comprising a wide range of plant species would therefore be required.

The application of DNA-related techniques in the quality control of herbs is also limited by the fact that the DNA fingerprint of a plant is the same for the whole plant, while the content of biomarker or marker compounds is often strongly dependent on the plant part. Moreover DNA fingerprinting might allow correct authentication of a plant but at present, it does not give information about the concentration of active constituents or marker compounds. A combination of DNA-related and phytochemical techniques is necessary for quality control applications. Finally, DNA-based techniques generally cannot be used for preparations containing herbal extracts, even though the successful authentication of herbal starting materials based on DNA extracted from small amounts of plant cells which are still present in herbal extracts and tinctures has been reported. However, more experience is required to evaluate the general feasibility of such a technique.

DNA-related techniques are a valuable complement, but are suggested to be done in addition to macroscopic, microscopic and phytochemical methods, and would be of use especially in raw herbs with many species and those cases which are generally substituted.

2.2.18. Transfusion and Infusion Assemblies and Similar Medical Devices

Sterile and Pyrogen free assemblies or devices in contact directly or indirectly with the cardiovascular system, the lymphatic system, or cerebrospinal fluid includes solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, implants extracorporeal oxygenator tubings and accessories, dialysers and dialysis tubing and accessories, heart valves, vascular grafts, intramuscular drug delivery catheters, and transfusion and infusion assemblies.

NOTE—These attributes do not apply to orthopedic products, latex gloves, or wound dressings.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Bacterial Endotoxins (2.2.3). Not more than 20.0 EU per medical devices and not more than 2.15 EU per medical devices for those medical devices in contact with the cerebrospinal fluid.

Preparation of test solution for bacterial endotoxin - Take not less than 3 and not more than 10 devices. Rinse or soak the devices with LAL Reagent Water. The volume of rinsing or extracting solution may be adjusted for the size and configuration of the device. For devices labeled "nonpyrogenic fluid pathway," flush the fluid pathway with extracting fluid at 37 ± 1° for one hour. Extracts may be combined, where appropriate. The endotoxin limit for the rinsing or extracting solution is calculated by the formula:

$$\frac{K \times N}{V}$$

Where K is equal to the amount of endotoxin per device, N is equal to the number of devices tested, and V is equal to the total volume of the extract or rinse.

If the undiluted rinsing or extracting solution is not suitable for Bacterial Endotoxins Test, repeat the inhibition or enhancement test after neutralization and removal of the interfering substances or after the solution has been diluted by a factor not exceeding the Maximum Valid Dilution. The Maximum Valid Dilution for devices is calculated by dividing the endotoxin limit by the labeled sensitivity 'λ' of the LAL reagent used.

Pyrogen (2.2.8). Complies with the test for Pyrogen for devices that cannot be tested by the Bacterial Endotoxins Test. Take 10 devices and rinse each with not more than 40 ml sterile saline and obtain a pooled effluent, utilizing preparation methods appropriate to the device as directed for Bacterial Endotoxins.

Other tests. The portions of medical devices that are made of plastics or other polymers meet the requirements specified for *Biological Tests- Plastics Containers for Parenteral Preparations* (6.2.1.1) and *Closures for Containers of Parenteral Products* (6.3).

2.2.19. Amino Acid Analysis

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organised as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify proteins and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect typical amino acids that might be present in a protein or peptide. It is necessary to hydrolyse a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatised for analysis.

Apparatus

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatography capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have post-column derivatisation capability, unless the sample is analysed using precolumn derivatisation. The detector is usually an ultraviolet/visible or fluorescence detector depending on the derivatisation method used. A recording device (e.g., integrator) is used for transforming the analogue signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

General Precautions

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight. Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipettes according to a maintenance schedule. Keep pipette tips in a covered box; the analysts may not handle pipette tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine. A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

Reference Material

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analysed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

Calibration of Instrumentation. Calibration of amino acid analysis instrumentation typically involves analysing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analysed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results. 4 to 6 amino acid standard levels are analysed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nanomole of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nanomoles of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

Repeatability

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes that it is necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analysing many replicates (e.g., 6 analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. The amino acid composition of a standard protein (e.g., bovine serum albumin) is often analysed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

Sample Preparation. Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, and detergents) can interfere with the amino acid analysis and are removed from the sample

before analysis. Methods that utilise post-column derivatisation of the amino acids are generally not affected by buffer components to the extent seen with pre-column derivatisation methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labour. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase High Performance Liquid Chromatography (HPLC) system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., *acetone*); (5) gel filtration.

Internal Standards. It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis, whose rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis; its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid as internal standards include *norleucine, nitrotyrosine,* and *α-aminobutyric acid.*

Protein Hydrolysis

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in *1 M hydrochloric acid* or soak tubes in concentrated nitric acid or in a mixture of equal volumes of concentrated *hydrochloric acid* and *nitric acid.* Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade *methanol,* dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hour may also be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolysing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids: tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine the amido bonds of Ile- Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described is used to address these concerns. Some of the hydrolysis techniques described (i.e., Methods IV-XI) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24 hour, 48 hour and 72 hour) is often employed to analyse the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (e.g., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for

each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

Method I

Acid hydrolysis using *hydrochloric acid* containing *phenol* is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of *phenol* to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution. 6 *M hydrochloric acid* containing 0.1 per cent to 1.0 per cent of *phenol*.

Procedure

Liquid phase hydrolysis. Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µl of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vaccum. Samples are typically hydrolysed at 110° for 24 hour in vacuum or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 hour and 72 hour) are investigated if there is a concern that the protein is not completely hydrolysed.

Vapour phase hydrolysis. This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24 hour hydrolysis time. Acid vapour hydrolyses the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample in vaccum to remove any residual acid.

Method II

Tryptophan oxidation during hydrolysis is decreased by using *mercaptoethanesulfonic* acid as the reducing acid.

Hydrolysis solution. *2.5 M mercaptoethanesulfonic acid solution.*

Vapour phase hydrolysis. Dry about 1 µg to 100 µg of the protein/peptide under examination in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 µl of the hydrolysis solution. Seal the larger tube in vaccum (about 50 µm of mercury or 6.7 Pa) to vaporise the hydrolysis solution. Heat the hydrolysis tube to 170-185° for about 12.5 minutes. After hydrolysis, dry the hydrolysis tube in vaccum for 15 minutes to remove the residual acid.

Method III

Tryptophan oxidation during hydrolysis is prevented by using *thioglycollic acid* as the reducing acid.

Hydrolysis solution. *7 M hydrochloric acid* containing 1 per cent *phenol*, 10 per cent of *trifluoroacetic acid and* 20 per cent of *thioglycollic acid.*

Vapour phase hydrolysis. Dry about 10 µg to 50 µg of the protein/peptide under examination in a sample tube. Place the sample tube in a larger tube with about 200 µl of the hydrolysis solution. Seal the larger tube in vaccum (about 50 µm of mercury or 6.7 Pa) to vaporise the *thioglycolic acid*.Heat the sample tube to 166° for about 15-30 minutes. After hydrolysis, dry the sample tube in vaccum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method IV

Cysteine/cystine and methionine oxidation is performed with *performic acid* before the protein hydrolysis.

Oxidation solution. Use *performic acid* freshly prepared by mixing 1 volume of *hydrogen peroxide solution* (30 per cent) and 9 volumes of *anhydrous formic* acid and incubating at room temperature for 1hour.

Procedure. Dissolve the protein/peptide sample in 20 µl of anhydrous formic acid and heat at 50° for 5 minutes then add 100 µl of the oxidation solution. Allow the oxidation to proceed for 10-30 minutes. In this reaction, cysteine is converted to *cysteic acid* and *methionine* is converted to methionine-sulfone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method I or Method II. This technique may cause modifications to tyrosine residues in the presence of halides.

Method V

Cysteine/cystine oxidation is accomplished during the liquid phase hydrolysis with *sodium azide.*

Hydrolysis solution. To *6 M hydrochloric acid* containing 0.2 per cent of *phenol*, add *sodium azide* to obtain a final concentration of 2 g per litre. The added *phenol* prevents halogenation of tyrosine.

Liquid phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110° for 24 hours. During the hydrolysis, the cysteine/cystine present in the sample is converted to

cysteic acid by the *sodium azide* present in the hydrolysis solution. This technique allows better tyrosine recovery than Method IV, but it is not quantitative for *methionine*. Methionine is converted to a mixture of the parent *methionine* and its 2 oxidative products, *methionine-sulfoxide* and *methionine-sulfone*.

Method VI

Cysteine/cystine oxidation is accomplished with *dimethyl sulfoxide*.

Hydrolysis solution. To *6 M hydrochloric acid* containing 0.1 per cent to 1.0 per cent of *phenol*, add *dimethyl sulfoxide* to obtain a final concentration of 2 per cent v/v.

Vapour phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110° for 24 hours. During the hydrolysis, the cysteine/cystine present in the sample is converted to *cysteic acid* by the *dimethyl sulfoxide* present in the hydrolysis solution. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the *cysteic acid* recovery from oxidative hydrolysis of standard proteins containing 1 to 8 mol of *cysteine*. The response factors from protein/peptide hydrolysates are typically about 30 per cent lower than those for non- hydrolysed cysteic acid standards. Because *histidine*, *methionine*, *tyrosine*, and *tryptophan* are also modified, a complete compositional analysis is not obtained with this technique.

Method VII

Cysteine/cystine reduction and alkylation is accomplished by a vapour phase pyridylethylation reaction.

Reducing solution. Transfer 83.3 µl of *pyridine*, 16.7 µl of *4-vinylpyridine*, 16.7 µl of *tributylphosphine*, and 83.3 µl of *water* to a suitable container and mix.

Procedure. Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the reducing solution to the large tube, seal in vaccum (about 50 µm of mercury or 6.7 Pa), and heat at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated sample can then be acid hydrolysed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1to 8 mol of cysteine to evaluate the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

Method VIII

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock solutions. Prepare and filter 3 solutions: *1 M Tris-hydrochloride* pH 8.5 containing *4 mM disodium edetate* (stock solution A), *8 M guanidine hydrochloride* (stock solution B), and 10 per cent of *2-mercaptoethanol* (stock solution C).

Reducing solution. Prepare a mixture of 1 volume of stock solution A and 3 volumes of stock solution B to obtain a buffered solution of 6 M *guanidine hydrochloride* in 0.25 M *tris-hydrochloride*.

Procedure. Dissolve about 10 µg of the test sample in 50 µl of the reducing solution, and add about 2.5 µl of stock solution C. Store under nitrogen or argon for 2 hour at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µl of *4-vinylpyridine* to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in vacuum centrifuge before acid hydrolysis.

Method IX

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock solutions. Prepare as directed for Method VIII.

Carboxymethylation solution. Prepare a 100 g per litre solution of *iodoacetamide* in *ethanol*.

Buffer solution. Use the reducing solution, prepared as described for Method VIII.

Procedure. Dissolve the test sample in 50 µl of the buffer solution, and add about 2.5 µl of stock solution C. Store under *nitrogen* or *argon* for 2 hour at room temperature in the dark. Add the *carboxymethylation solution* in a ratio 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. If the thiol content of the protein is unknown, then add 5 µl of *100 mM iodoacetamide* for every 20 nmol of protein present. The reaction is stopped by adding excess of *2-mercapto-ethanol*. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethyl-cysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

Method X

Cysteine/cystine is reacted with *dithiodiglycolic acid* or *dithiodipropionic acid* to produce a mixed *disulfide*. The choice of *dithiodiglycolic* acid or *dithiodipropionic* acid depends on the required resolution of the amino acid analysis method.

Reducing solution. A 10 g per litre solution of *dithiodiglycolic acid* (or *dithiodipropionic acid*) in *0.2 M sodium hydroxide*.

Procedure. Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µl of the reducing solution. Add 10 µl of *isopropyl alcohol*, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolysed using Method I. This method has the advantage that other amino acid residues are not derivatised by side reactions, and that the sample does not need to be desalted prior to hydrolysis.

Method XI

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and *glutamic acid* residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1- trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to *diaminopropionic acid* and *diaminobutyric acid* residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing solutions. Prepare and filter 3 solutions: a solution of *10 mM trifluoroacetic acid* (Solution A), a solution of *5 M guanidine hydrochloride* and *10 mM trifluoroacetic acid* (Solution B), and a freshly prepared solution of *dimethyl-formamide* containing 36 mg of *bis(1,1- trifluoroacetoxy) iodobenzene BTI* per ml (Solution C).

Procedure. In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 ml of Solution A or Solution B and 2 ml of Solution C. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hour in the dark. The sample is then dialysed with water to remove the excess reagents. Extract the dialysed sample 3 times with equal volumes of butyl acetate, and then lyophilise. The protein can then be acid hydrolysed using previously described procedures. The *diaminopropionic and diaminobutyric acid* residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatised and BTI-derivatised acid hydrolysis. The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatisation; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.

Methodologies of Amino Acid Analysis: General Principles

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by post-column derivatisation (e.g., with *ninhydrin* or *o-phthalaldehyde*). Post-column derivatisation techniques can be used with samples that contain small amounts of buffer components, (such as salts and urea) and generally require between 5 µg and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve pre-column derivatisation of the free amino acids (e.g., *phenyl isothio-cyanate; 6-aminoquinolyl-N- hydroxysuccinimidyl carbamate* or *o-phthalaldehyde*; (dimethylamino) *azobenzenesulfonyl chloride; 9-fluorenylmethyl chloroformate*; and *7- fluoro-4-nitrobenzo-2-oxa-1,3-diazole*) followed by reversed-phase HPLC. Pre-column derivatisation techniques are very sensitive and usually require between 0.5 µg and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Pre-column derivatisation techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Post-column derivatisation techniques are generally influenced less by performance variation of the assay than pre-column derivatisation techniques.

Method I - Post-column Ninhydrin Derivatisation

Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simple amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has a characteristic purple or yellow colour. Amino acids, except imino acid, give a purple colour, and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour, and show an absorption maximum at 440 nm. The post-column reaction between ninhydrin and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition. The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for the proline derivative. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Method II - Post-column OPA derivatisation

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to produce highly fluorescent isoindole products. This reaction is used for the post-column derivatisation in analysis of amino acids by ion-exchange chromatography. The principal of the separation is the same as Method I.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with *sodium hypochlorite* or *chloramine T* allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by post- column oxidation with *sodium hypochlorite* or *chloramine T* and post-column derivatisation using OPA and a thiol compound such as *N-acetyl-L-cysteine* or *2- mercaptoethanol*. The derivatisation of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite or chloramine T. Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After post-column derivatisation of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of picomole level for most of the OPA-derivatised amino acids. Response linearity is obtained in the range of a few picomole levels to a few tens of nanomole level. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

Method III - Pre-column PITC Derivatisation

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 254 nm. Therefore, pre-column derivatisation of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyse the amino acid composition.

After the reagent is removed under vacuum, the derivatised amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after 3 days.

Separation of the PTC-amino acids on a reversed-phase HPLC with an octadecylsilane (ODS) column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the PTC-amino acids. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

Method IV - Pre-column AQC Derivitisation

Pre-column derivatisation of amino acids with *6-aminoquinolyl-N-hydroxysuccinimidyl carbamate* (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase High-Pressure Liquid Chromatography. Therefore, pre-column derivatisation of amino acids with AQC followed by reversed-phase HPLC separation with fluorimetric detection is used to analyse the amino acid composition.

Separation of the AQC-amino acids by a reversed-phase HPLC with an ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, *6-aminoquinoline*. Excess reagent is rapidly hydrolysed ($t_{1/2}$ < 15 s) to yield *6-aminoquinoline*, *N-hydroxysuccinimide* and *carbon dioxide*, and after 1 minute no further derivatisation can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature. Therefore AQC-amino acids have more than sufficient stability to allow for overnight, automated chromatographic analysis.

The detection limit is considered to range from about 40 fmol to 320 fmol for each amino acid, except for *cystein*. The detection limit for *cystein* is approximately 800 fmol. Response linearity is obtained in the range of 2.5-200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatised protein hydrolysates derived from as little as 30 ng of protein/peptide.

Method V - Pre-column OPA Derivatisation

Pre-column derivatisation of amino acids with *o-phthalaldehyde* (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline). OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. *2-Mercaptoethanol* or *3-mercaptopropionic acid* can be used as the thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the

rapid kinetics for the reaction, make it amenable to automated derivatisation and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in Method VII or Method VIII.

Pre-column derivatisation of amino acids with OPA is followed by a reversed-phase High-Pressure Liquid Chromatography separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatisation. The liquid chromatography equipped with a fluorometric detector is used for the detection of derivatised amino acids. Fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol per ml via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol per ml.

Method VI - Pre-column DABS-Cl Derivatisation

Pre-column derivatisation of amino acids with *(dimethylamino)azobenzenesulfonyl chloride (DABS-Cl)* followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labelling of amino acids. Amino acids labelled with DABS-Cl (DABS-amino acids) are highly stable and show an absorption maximum at 436 nm.

DABS-amino acids, all naturally occurring amino acid derivatives, can be separated on an ODS column of a reversed-phase (HPLC) by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyse the imino acids such as proline together with other amino acids at the same degree of sensitivity, DABS-Cl derivatisation method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with *sulfonic acids* such as *mercaptoethanesulfonic acid, p- toluenesulfonic acid or methanesulfonic acid* described in Method 2 under Protein hydrolysis. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and *diaminobutyric acid*, respectively, by treatment of protein/peptide with BTI described in Method XI under Protein hydrolysis.

The non-proteinogenic amino acid norleucine cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol per ml. As little as 2-5 pmol per ml of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10-30 ng per ml of the dabsylated protein hydrolysate is required for each analysis.

Method VII - Pre-column FMOC-Cl Derivatisation

Pre-column derivatisation of amino acids with *9-fluorenylmethyl chloroformate* (FMOC-Cl) followed by reversed-phase (High-Pressure Liquid Chromatography) HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction proceeds under mild conditions in aqueous solution and is completed in 30 second. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of 10 volumes of acetonitrile, 40 volumes of *methanol* and 50 volumes of *acetic acid buffer* to a mixture of 50 volumes of *acetonitrile* and 50 volumes of *acetic acid buffer* and 20 amino acid derivatives are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm. The detection limit is in the low femtomole range. A linearity range of 0.1-50 µM is obtained for most of the amino acids.

Method VII - Pre-column NBD-F Derivatisation

Pre-column derivatisation of amino acids with *7-fluoro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-F)* followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatised with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing a gradient elution system consisting of *acetonitrile* and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 minutes. *Aminocaproic acid* can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for the pre-column OPA derivatisation method (Method V), excluding

proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis can be achieved with about 1.5 mg of protein hydrolysates in the pre-column reaction mixture.

Calculation

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapour phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Amino Acid Mole Percent. This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular mass of the protein under investigation is unknown. This information can be used to corroborate the identity of a protein/peptide and has other applications. Carefully identify and integrate the peaks obtained as directed for each procedure. Calculate the mole percent for each amino acid present in the test sample using the formula:

$$\frac{100\, r_u}{r}$$

in which r_u is the peak response, in nanomoles, of the amino acid under test; and r is the sum of peak responses, in nanomoles, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under examination to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples. This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in micrograms, of each recovered amino acid using the formula:

$$\frac{mM_r}{1000}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; and M_r is the average molecular mass for that amino acid, corrected for the mass of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analysed after appropriate correction for partially and completely destroyed amino acids. If the molecular mass of the unknown protein is available (i.e., by SDS- PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid using the formula:

$$\frac{m}{\left(\frac{1000M}{M_n}\right)}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; M is the total mass, in micrograms, of the protein; and M_n is the molecular mass of the unknown protein.

Known protein samples. This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular mass and amino acid composition using the amino acid analysis data. When the composition of the protein being analysed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine) and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nanomoles, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically greater than 5 per cent variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, using the formula:

$$\frac{1000m}{m_s}$$

in which m is the experimentally determined quantity, in nanomoles per amino acid residue, of the amino acid under test; and m_S is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

2.2.20. Maintenance, Identification, Preservation and Disposal of Microorganisms

The methods of maintenance, preservation and identification of Microbial Reference Cultures used in microbiological tests, are very crucial for quality testing of samples. Microbial cultures are most delicate standards to handle because their viability and characteristics are dependent on proper handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the chance of contamination or alteration of growth characteristics. Microbial Reference Cultures used in testing must be acquired from an authenticated and recognised national or international culture collection centre. There are several National and International Culture Centres. Some of them are given below.

- MTCC (Microbial Type Cultures Collection and Gene Bank, Institute of Microbial Technology, Chandigarh)
- ATCC (American Type Cultures Collection Centre, Maryland, U.S.A)
- NCTC (National Collection of Type Culture, London)
- NCIM (National Collection of Industrial Microorganism, NCL, Pune)

Microbial cultures can be obtained in lyophilized or other standard form with proper labelling of passage number and other essential requirements. Confirmation of the purity and identity of the culture should performed before it is used in testing. A list of Microbial Reference Culture used in various microbiological testing is given in Table 1.

Maintenance of Microbial Reference Cultures

The maintenance of Microbial Reference Cultures is an essential requirement to maintain their viability and characteristics. Actively growing cultures are required for quality control, comparative testing, for bioassays, vaccines and for various other reasons. The original culture from a national or international culture collection is revived and grown in an appropriate medium as per the instruction of the supplier or a validated method. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryo protective medium, transferred to vials and frozen at –30° or below and stored, until use. If lyophilised or kept in liquid nitrogen then it may survive for much longer time. These frozen stocks can then be used to inoculate monthly or weekly working cultures as per requirements. Once opened, unused cell suspension should not be refreezed. The unused portion should be discarded to minimise the risk of viability and contamination of the stock. In order to prevent any phenotypic changes in the strains used, the organisms used should not be more than 5 passages from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium. Schematic flow diagram of passages of Microbial Reference Cultures is given in Fig. 2.2.20-1.

The working cultures should be stored in a refrigerator (2° to 8°), which retards the growth, protects from the damage due to evaporation of medium and preserves the culture.

Identification of Microbial Cultures

Proper identification and characterisation is needed for authentication of microbial strains. Confirmation of identity for commonly used laboratory organisms should be done at the level of genus and species. Such properly identified and authenticated strains should be used for various microbiological tests. Additionally, identification is also required to be carried out when organisms are recovered at high rate or in numbers that exceeds recommended levels for specific categories of products. It is useful in the assessment of contamination recovered from failed aseptic simulation (media fills test), when the aseptically filled samples fails in sterility test.

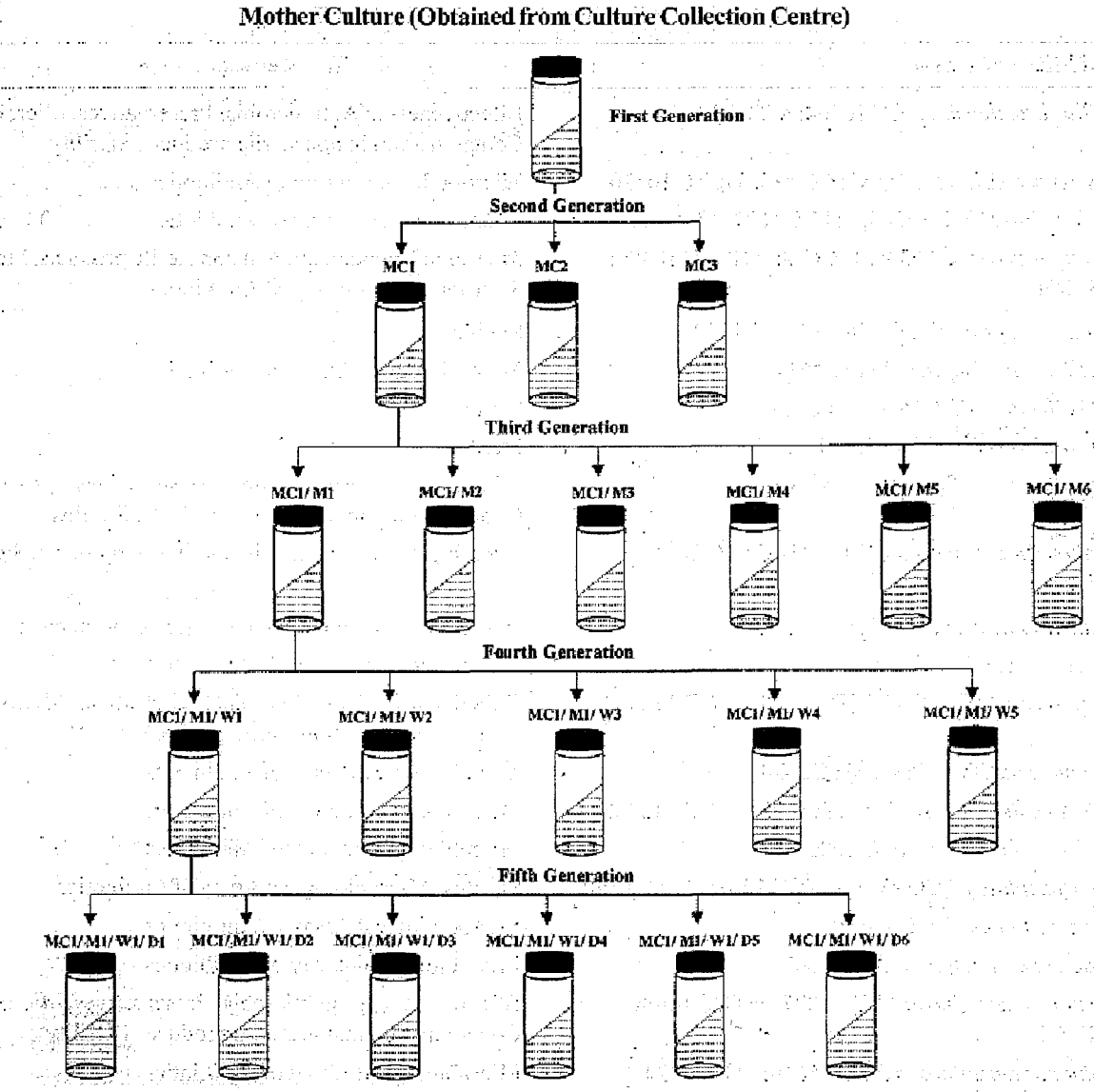
The first step in identification is to isolate a culture in purest form. This is typically accomplished by successive streaking of the culture of interest in a quadrant pattern on appropriate General Microbiological Solid Media with the objective of obtaining discreet colonies that yield pure cultures. This technique also allows phenotypic expression and growth of sufficient inoculum for succeeding identification procedures. The various methods of microbial identification are mentioned in Table 2. Microbiologists should recognise that expression of the microbial phenotype may be affected by isolate's origin, media selection and growth conditions. Therefore, the media for identification and repeated subculture may affect the results of phenotypic identification. In contrast, the microbial genotypic methods are generally well conserved and unaffected by culture techniques.

Phenotypic Identification

Morphological Characterisation.. Routine characterisation of microorganisms may include the determination of colony

Table 1– List of Microbial Cultures Required For Testing

Name of Micro-organism	Test Methods
Aspergillus brasilensis ATCC 16404; MTCC1344	Effectiveness of Antimicrobial Preservatives, Microbial Contamination in non sterile products, Sterility
Bacillus cereus ATCC 11778; MTCC 430; NCTC 10320	Microbiological Assay of Antibiotics
Bacillus pumilus ATCC 14884; MTCC 1607; NCTC 8241	Microbiological Assay of Antibiotics
Bacillus subtilis ATCC 6633; MTCC 441; NCTC 10400; NCIMB 8054	Microbial Contamination in non sterile products, Sterility Microbiological Assay of Antibiotics
Bacteroides vulgatus ATCC 8482; MTCC 1350	Sterility
Bordetella bronchiseptica ATCC 4617	Microbiological Assay of Antibiotics
Candida albicans NCYC 854; MTCC 854	Sterility
Candida albicans ATCC 2091; MTCC 183	Sterility
Candida albicans, ATCC 10231; MTCC 227	Effectiveness of Antimicrobial Preservatives, Microbial Contamination in non sterile products, Sterility
Clostridium sporogenes ATCC 19404; MTCC 2684; NCTC 532	Microbial Contamination in non sterile products, Sterility
Clostridium spp. ATCC 11437; MTCC 1349	Microbial Contamination in non sterile products, Sterility
Entrococcus hirae ATCC 10541	Microbiological Assay of Antibiotics
Escherichia coli ATCC 8739; MTCC 1687; NCTC 12923	Effectiveness of Antimicrobial Preservatives, Microbial Contamination in non sterile products
Escherichia coli ATCC 9637; MTCC 448	Microbiological Assay of Antibiotics
Escherichia coli ATCC 10536; MTCC 739; NCTC 10418	Microbiological Assay of Antibiotics
Klebsiella pneumoniae ATCC 10031	Microbiological Assay of Antibiotics
Kocuria rhizophila ATCC 9341; MTCC 1541; NCTC 8340	Sterility, Microbiological Assay of Antibiotics
Micrococcus luteus ATCC 10240	Microbiological Assay of Antibiotics
Mycobacterium smegmatis ATCC 607	Microbiological Assay of Antibiotics
Pseudomonas aeruginosa ATCC 9027; MTCC 1688; NCTC 12924	Effectiveness of Antimicrobial Preservatives, Microbial Contamination in non sterile products, Sterility
Pseudomonas aeruginosa ATCC 25619; MTCC 424	Microbiological Assay of Antibiotics
Saccharomyces cerevisiae ATCC 2601	Microbiological Assay of Antibiotics
Saccharomyces cerevisiae ATCC 9763; MTCC 170; NCTC 10716	Microbiological Assay of Antibiotics
Salmonella enterica spp. enterica serotype abony NCTC 6017; MTCC 3858;	Microbial Contamination in non sterile products
Salmonella enterica spp. enterica serotype typhimurium ATCC 14028	Microbial Contamination in non sterile products
Shigella boydii ATCC 8700; MTCC 11947; NCTC 12985	Microbial Contamination in non sterile products
Staphylococcus aureus ATCC 6538; MTCC 737; NCTC 7447	Effectiveness of Antimicrobial Preservatives, Microbial Contamination in non sterile products, Sterility
Staphylococcus aureus ATCC 9144; MTCC 96; NCTC 6571	Microbiological Assay of Antibiotics
Staphylococcus aureus ATCC 29737	Microbiological Assay of Antibiotics
Staphylococcus epidermidis ATCC 12228; MTCC 3615	Microbiological Assay of Antibiotics
Zygosaccharomyces rouxii NCYC 381; MTCC 2635	Effectiveness of Antimicrobial Preservatives,



Where;

A)	MC1, MC2, MC3	-	Master Cultures
B)	MC1/M1 to MC1/M6	-	Monthly Cultures
C)	MC1/M1/W1 to MC1/M1/W5	-	Weekly Cultures
D)	MC1/M1/W1/D1 to MC1/M1/W1/D6	-	Daily Cultures

Fig. 2.2.20-1 : Schematic Flow Diagram of Passages of Microbial Cultures

morphology and cellular morphology. The shape, size and arrangements of microbial cell can be examined by microscopic tools. The staining techniques are another requirement to differentiate microorganisms. There are different types of microbiological staining techniques available such as Gram staining, Acid-fast staining, Spore staining, Flagella staining etc. The most commonly used stain is Gram stain. Based on the Gram staining, microorganisms can be divided into two major groups viz Gram-positive and Gram-negative. The acid-fast staining is useful for the identification of acid fast bacteria having large amounts of lipid substances within their cell walls called mycolic acids. The spores staining can be accomplished by using a malachite green stain for bacterial spores (endospores). The flagella staining are used to determine the

Table 2 – Various characteristics used in Microbial Identification

Categories	Characteristics
Culture	Colony morphology, colour, shape and size, pigment production etc.
Morphological	Cellular morphology, cell size, cell shape, flagella type, reserve material, Gram-staining, spore and acid-fast staining, mode of sporulation etc.
Physiological	Oxygen tolerance, pH range, temperature optimum and range, salinity tolerance etc.
Biochemical	Carbon utilization, carbohydrate oxidation or fermentation, enzyme patterns etc.
Inhibition	Bile salt-tolerance, antibiotic susceptibility, dye tolerance etc.
Serological	Agglutination, fluorescent antibody etc.
Chemo-taxonomic	Fatty acid profile, microbial toxins, whole cell composition etc.
Ecological	Origin of the organism etc.
Genotypic	DNA base ratio (G + C content), Genomic fingerprinting, DNA–DNA hybridization and 16S and 23S rRNA sequences and DNA probes etc.

presence, location and numbers of flagella possessed by the microorganisms.

Biochemical reactions. Species that cannot be distinguished by morphology and cultural characteristics may exhibit metabolic differences that can be exploited. It is usual to test the ability of the organism to produce acidic and gaseous end products, when presented with individual carbohydrates (glucose, lactose, sucrose, mannitol etc.) as the sole carbon source. Other biochemical test determines whether the bacterium produces particular end products (e.g. indole, hydrogen peroxide etc.) when grown on a suitable culture media and whether it possesses certain enzyme activities such as oxidase, catalase, urease, gelatinase etc.

Genotypic Identification

Genotypic methods have been shown to be precise and rapid than traditional phenotypic and biochemical techniques. Genotypic microbial identification methods are theoretically more reliable because nucleic acid sequences are highly conserved in most microbial species. These methods are more appropriate for the identification of microorganisms, found in pharmaceutical products at the species or genus level. Applicable genotypic method includes DNA base ratio (G+C content); DNA-DNA hybridization; 16S or 23S/ITS rRNA sequencing, genomic fingerprinting, multilocus sequence typing (MLST), pyrosequencing, DNA probes etc. These methods can be technically challenging for microbiologist and required skilled manpower to analyse the samples. Further, if strain level identification is required in the course of an investigation, the genotypic method and serotyping are the only major techniques. The details of several genomic techniques are described in DNA-Based Authentication Techniques (2.2.17).

Preservation of Microbial Reference Cultures

To preserve microorganisms, it is necessary to reduce their metabolism to a minimum level. There are several methods available for preservation of Microbial Cultures. The choice of the method to be used depends upon the purpose, size of collection and the facility available. Live cultures on a culture medium can be successfully stored for short time in refrigerators or cold rooms maintained at 2° to 8°. Generally the metabolic activities of the microorganism will be greatly slowed down at this temperature. Cultures can be preserved for longer time in glycerol at −80° in a deep freeze. Microorganisms can be preserved using liquid paraffin. In this procedure sterile liquid paraffin is poured over the slant culture of microorganisms till it completely covers the surface of the slant and remains at least two centimetres above the tip of the slant. The slant is then stored in upright position at room temperature or preferably at 2° to 8°. The paraffin prevents the dehydration of the medium and ensures anaerobic conditions. It slows the metabolic activity by reduced growth. Paraffin should be sterilised using a validated process. If autoclaved (at 121° for 45 minutes) care should be taken to ensure that moisture do not get mixed with the oil. It is preferable to sterilise the oil in hot air oven at 160° to 170° for one hour.

Both bacteria and yeast can be stored in silica gel powder at low temperature. The basic principle in this technique is quick desiccation at low temperature which allows the cell to remain viable for a longer time. In this procedure, finely powdered, dry heat sterilised and cooled silica powdered is mixed with a very thick suspension of cells in sterile conditions and stored at low temperature. Lyophilization is the best method of preservation of microorganisms. Lyophilization or freeze-drying is the process in which microorganisms are suspended

in a medium containing sodium glutamate or skimmed milk powder, sugar, serum etc. and frozen at low temperature (about - 40° to -70°) and then vacuum dried in the frozen condition (by sublimation of ice). The dried material is preserved in vacuum or under an inert gas such as nitrogen in hermetically sealed ampoules which are stored in dark at 2° to 8° in refrigerator. Cryopreservation in Liquid nitrogen at –196° also suspends the metabolism of cells and they survive unchanged for long periods. In this method, microbes are suspended in the presence of a stabilizing agent, such as glycerol or Dimethyl sulphoxide (DMSO), that prevents the formation of ice crystals which may kill frozen cells, are sealed into small ampoules and store in liquid nitrogen (-196°).

Disposal of Microbial Cultures

All microbial cultures to be discarded must be first sterilized, even if they are not pathogenic. Chemical disinfection, Steam sterilization, Incineration etc. are the practical methods by which microbial cultures may be sterilized.

Chemical disinfectants have a range of properties, and no single one is effective in all situations. The most common chemicals such as alcohols (70 per cent *ethanol* or 70 per cent *isopropyl alcohol*), Chlorine releasing compounds (e.g. *sodium hypochlorite*), Quaternary ammonium compounds, Phenolic compounds, *Iodine solution* are generally used for the decontamination of microbial cultures. A number of factors should be considered in choosing a chemical for decontamination because microorganisms vary in their resistance to chemical agents. Most disinfectant solutions need to be regularly prepared as fresh solutions to avoid growth of micro-organisms in the solution and to ensure optimum activity of the disinfectant chemical. The chemical disinfection has many limitations and hence, it is least preferred method.

The most popular method of discarding microbial cultures is steam sterilization. The most common method of steam sterilization is autoclaving. Materials containing viable microorganism (e.g. Petri-plates, culture tubes, liquid media tubes etc.) should be placed in a leak-proof container or autoclavable plastic bags and autoclaved at higher temperatures which ensure more rapid killing. The usual standard temperature/pressure employed is 121°/15 psi for 20 minutes. However, longer times are needed for larger culture media loads, large volumes of liquid and denser materials. After autoclaving the material may be disposed off.

Incineration is preferred for pathogens isolated from clinical specimens. Materials for incineration, even with prior decontamination, should be transported to the incinerator in bags, preferably plastic. Incineration is carried out in specially designed furnaces that achieve high temperatures and are constructed to be airtight. The temperature needed for the incineration should be at least 800°.

2.2.21. Glycoprotein and Glycan Analysis

Overview

A number of glycoprotein drugs have been developed as a result of advances in biotechnology, and many naturally derived protein drugs possess complex glycan structures. Glycosylation, a posttranslational modification of these proteins, can play an important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these agents. The two main types of protein glycosylation are N-glycosylation and O-glycosylation. Unlike transcription and translation, glycosylation is not a template-driven process; therefore variability in the glycosylation pattern of a protein can arise, caused by different sources or different manufacturing processes. Differences in this pattern are known to affect biological activity. Glycosylation patterns may therefore be an important set of attributes that arise in characterizing a candidate glycoprotein intended for therapeutic use and in ensuring its stability and quality.

The first part of this chapter provides a brief introduction to glycobiology and describes the complexity of glycan structures. The subsequent parts provide flow charts and a series of general analytical strategies that can be used to characterize glycoprotein glycans by means of the following:

1. Direct analysis of glycoproteins; and
2. Analysis of released non-derivatized or derivatized glycans by various methods of chromatographic and electrophoretic separation and mass spectrometry (MS).

Different approaches to analyzing monosaccharides are described at the end of the chapter.

Protein Glycosylation

Most proteins in eukaryotic cells undergo glycosylation and other posttranslational modifications before being trafficked to lysosomes, becoming membrane bound at the cell surface, or being secreted. Glycosylation varies significantly from cell to cell, tissue to tissue, and species to species because of the varying expression of hundreds of glycosyltransferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum (ER).

Four main types of enzymatic glycosylation are found in proteins:

1. N-Glycosylation, which involves the initial transfer of oligosaccharides to the nitrogen on the terminal amide group of asparagine and their subsequent processing and modification to a series of glycan chains;
2. O-Glycosylation, which in general involves the initial transfer of monosaccharides to the hydroxyl groups of serine and threonine and subsequent elongation and

branching of the saccharide chain by the addition of monosaccharides;

3. Glycosylphosphatidylinositol (GPI) anchor, which is a glycolipid linked to the C-terminus of a protein; and
4. C-Glycosylation, which involves the formation of a carbon–carbon bond between the C2 carbon of the indole ring of tryptophan and the C1 carbon of α-mannopyranosyl residue.

Any given protein may contain multiple N-, O-, or C-glycosylations, but not more than one GPI anchor. A non-enzymatic addition of saccharides, called glycation, can occur when proteins are mixed with reducing sugars via a complex series of reactions. The two protein glycosylation types that are generally of concern and that are analyzed in glycoprotein drug substances are N- and O-glycosylation. Each of these is discussed below.

N-Glycosylation

The biosynthesis of N-glycans in glycoproteins can be described as a four-step process:

1. Lipid-linked glycan chain initiation and elongation;
2. Transfer of oligosaccharide to the protein or nascent polypeptide chain;
3. Processing of the N-glycan chain by removal of specific glucose and mannose residues; and
4. Modification of the N-glycan chain by the addition of residues to the non-reducing ends of the glycan chain.

The consensus amino acid sequence for N-glycosylation is Asn–Xaa–Thr/Ser (where Xaa is any amino acid other than proline). Overall, only about two-thirds of all potential sequences, termed sequons, are glycosylated, and currently there is no method to predict which sequon will be glycosylated. The role of protein N-glycosylation is usually protein trafficking and secretion.

N-glycans can be categorized as high-mannose, hybrid, or complex, depending on the extent of processing (Fig.2.2.21-1). High-mannose structures (Appendix 1) lack galactose or N-acetylglucosamine (GlcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both substituted GlcNAc residues and terminal mannose residues are present in the antennae, whereas complex structures have both α-1,6- and α-1,3-mannose residues substituted with GlcNAc moieties. Hybrid and complex glycans can exist with two or more branches, frequently termed antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary N-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (neuraminic acid) residues. Sialylation has been

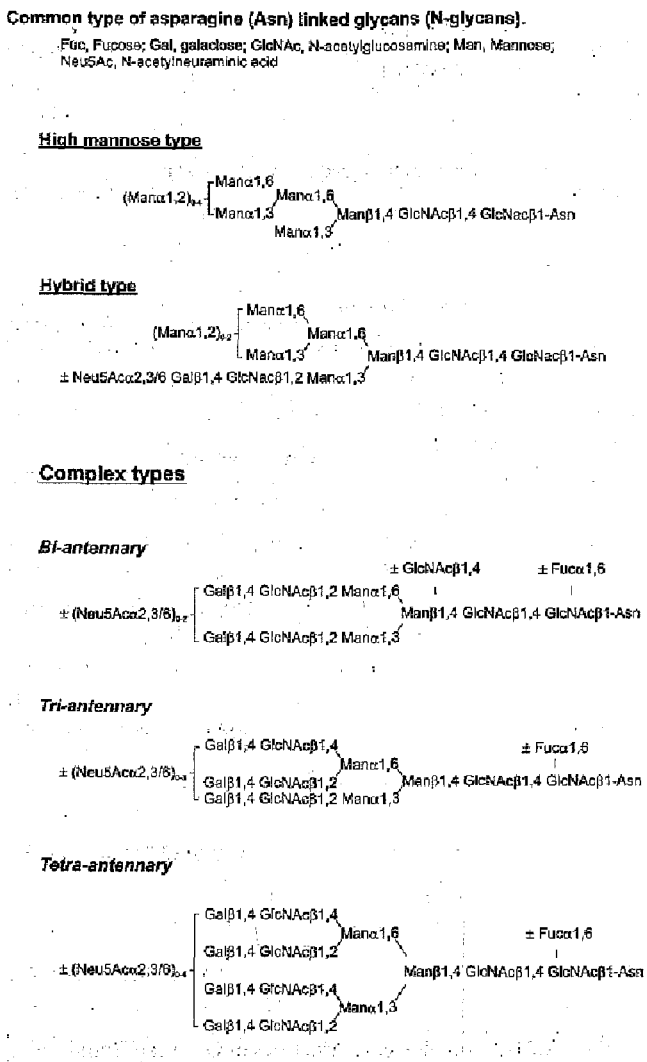


Fig.2.2.21-1 Common types of N-glycans. For abbreviations see Appendix 1

shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

O-Glycosylation

O-Glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and isomeric linkage of monosaccharides show greater variety than that in N-glycans, and at least eight different types have been identified (Fig.2.2.21-2). Although no consensus amino acid sequence for O-glycosylation has been determined, glycosylation is usually favored by the presence of proline one residue before or three residues after the glycosylation site and the absence of charged amino acid residues proximal to serine or threonine. The disaccharide

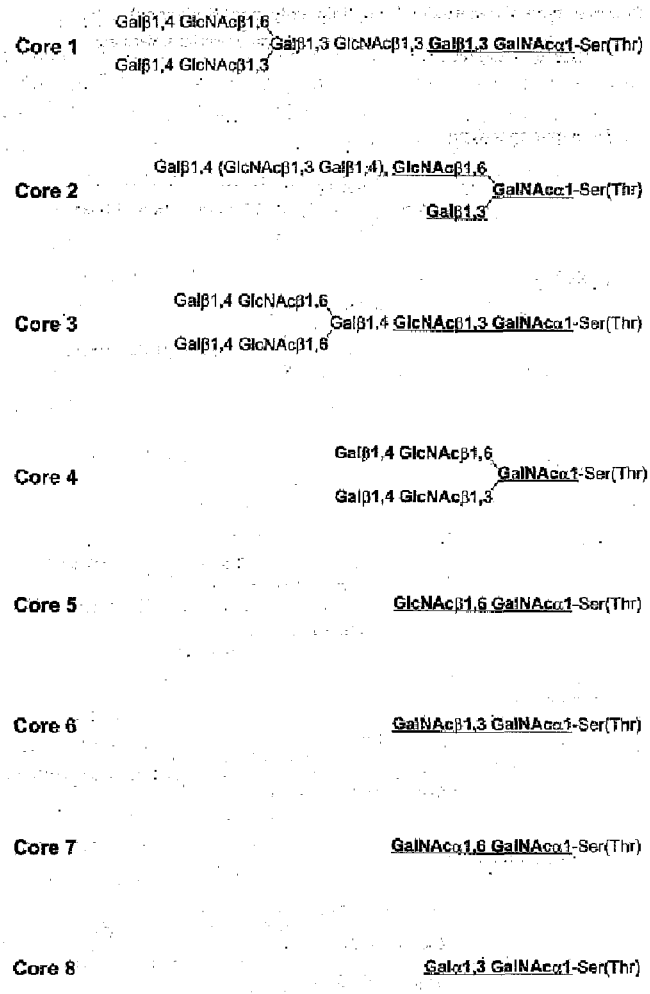


Fig.2.2.21-2 Common core structures of O-glycans (bold and underlined). For abbreviations see Appendix 1

unit N-acetyllactosamine, Galβ$_1$,4GlcNAc, is the most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

O-Glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other O-glycosylation, such as O-GlcNAc, is found on many nucleocytoplasmic proteins; and O-Man–linked glycosylation is found in some muscular and neural glycoproteins and in yeast. O-Fuc– and O-Glc–linked glycosylation types are found on many epidermal growth factor-like proteins that are associated with the Notch signaling pathway.

Glycan Heterogeneity

Not only the type of glycosylation (N- or O-linked), site occupancy, and the site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branching and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including cell-specific and growth-dependent availability of glycosyltransferases and exo-glycosidases found in the Golgi bodies and ER. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity. The control and maintenance of a consistent glycoform profile during glycoprotein manufacturing is still a considerable challenge, due to the high variability inherent to the process of glycosylation and to the variability introduced by several culture and process parameters (e.g. expression system, culture conditions), whose mechanisms are still under investigation and hard to control. The factors influencing glycan heterogeneity can be classified into two broad categories as variation due to host cell expression system and variation due to environmental factors and culture conditions.

Host-Cell Expression Systems and Glycosylation

Bacteria

Although both O- and N-glycosylation have been shown to occur in a variety of prokaryotes, *Escherichia coli*, the bacterium of choice for many therapeutic products, does not produce glycosylated proteins.

Yeast

Yeast produces both N-glycosylated and O-glycosylated proteins. In yeast hypermannosylation with the N-glycan chain that contains more than 100 mannose residues can occur, but sialylation does not occur unless the organism is genetically modified. The development of recombinant strains of *Pichiapastoris* that contain inserted heterologous genes for various glycosylation enzymes has allowed the humanization of N-glycosylation pathways in this yeast. O-Glycosylation in yeast is also significantly different from that in mammalian cells. In contrast to mammalian cells, serine or threonine O-glycosylation is linked via mannose and often consists of linear chains of as many as six mannose residues.

Insect cells

N-glycan chains of insect cells usually are of the high-mannose, trimannose or paucimannose, and truncated complex types (see Appendix 1 for definitions). Insect cells also produce glycoproteins bearing the Fuc α-1,3 residue linked to the proximal GlcNAc residue in the core chitobiose. This core fucose residue is a potent immunogen and allergen. O-Glycosylation in insect cells has not been well-studied, and although O-linked GalNAc–Ser(Thr) residues have been found, very few are processed further beyond the Galβ$_1$,3GalNAc–

Ser (Thr) sequence. Sialic acid residues have not been found on proteins produced in insect cells.

Plants and plant cells

Plant N-glycans contain mainly oligosaccharides of the oligomannose type, but also present are hybrid and truncated complex types of structures, with or without Xylβ1,2 attached to the β-linked mannose residue of the trimannosyl core and Fuc α-1,3 attached to the proximal GlcNAc residue of the core chitobiose. Both the Fuc and Xyl residues are immunogenic and have been shown to be part of the glyco-epitopes of several plant allergens. O-Glycosylation in plants has not been well studied but is known to consist predominantly of the addition of arabinogalactanchainsattached to hydroxyproline, threonine, and serine residues that are located in the plant cell wall or on the outer surface of the plasma cell membrane. These glycans are immunogenic.

Animal cells

The majority of glycosylated therapeutic proteins are produced in continuous animal cell lines. Chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), and mouse myeloma (SP2/0 or NS0) cells have all been employed. These animal cells generally produce proteins with humanlike glycosylation. Although there are several differences in glycosylation between rodent and human cells, such as the presence of N-glycolylneuraminic acid not found in humans, CHO cells have become a workhorse of the biotechnology industry.

Environmental factors and culture conditions

Several parameters of the cell culture environment are manipulated during therapeutic production to optimize cell growth and product yield. These parameters can also change the protein glycosylation. The parameters may be divided into the following categories: medium and nutrients, culture conditions, technology platform, and cell-related factors.

The culture medium determines the cell growth environment, which has a crucial influence on product quality. The various medium components like glucose, glutamine and other supplements in basal medium components have significant effect on glycans produced. Several culture parameters that influence cell environment, such as dissolved oxygen (DO), pH, carbon dioxide (CO_2) and temperature, are optimized to maximize the product yield, which has also been shown to affect protein glycosylation. DO have shown to influence glycosylation, apparently in a cell line- and/or protein-specific manner, generally affecting galactosylation. The external culture pH may change the internal Golgi pH, influencing the activities of key glycosylating enzymes and thus changing the protein glycan profile. pH affects specifically galactosylation, sialylation and glycan occupancy. The technology platform (bioreactor and mode of culture) can have a pronounced effect on the resulting glycoform profile of the protein The culture conditions mentioned above affect cell metabolism, in particular growth rate, density, viability and specific productivity. These cell-specific parameters have been shown to modify the glycosylation pathway by affecting the levels of extracellular glycosidases in the medium, which can step-wise remove monosaccharides from the glycans.

Glycan analysis for glycosylated biological drugs

Glycosylation of proteins may affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may be also be important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physicochemical testing of production batches to ensure batch-to-batch consistency. Glycosylation analysis may be appropriate for the following:

1. Characterizing the structure and stability of novel products and their stability to processing steps and storage;
2. Batch release testing and in process control testing; and
3. Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function underpins decisions about the information required at each development stage. For biological/biotechnological drug substances, the characterization criteria and specifications for batch release are generally set forth in the guidelines ICH Q6B, Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products; and ICH Q5E, Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process. Numerous approaches and methodologies are applied for glycan mapping. This variety is a consequence of the diversity and complexity of glycan structures and the available technology and detection systems.

Because of the diversity and complexity of glycan structures and the increasing availability and improvement of various detection systems and technology, analytical methods are wide ranging. Different methods that support step-by-step procedures depend on the glycoproteins, the availability of equipment, the expertise of individual scientists and groups, and the information required. The two most studied types of protein glycosylation that affect bioactivity are N- and O-glycosylation.

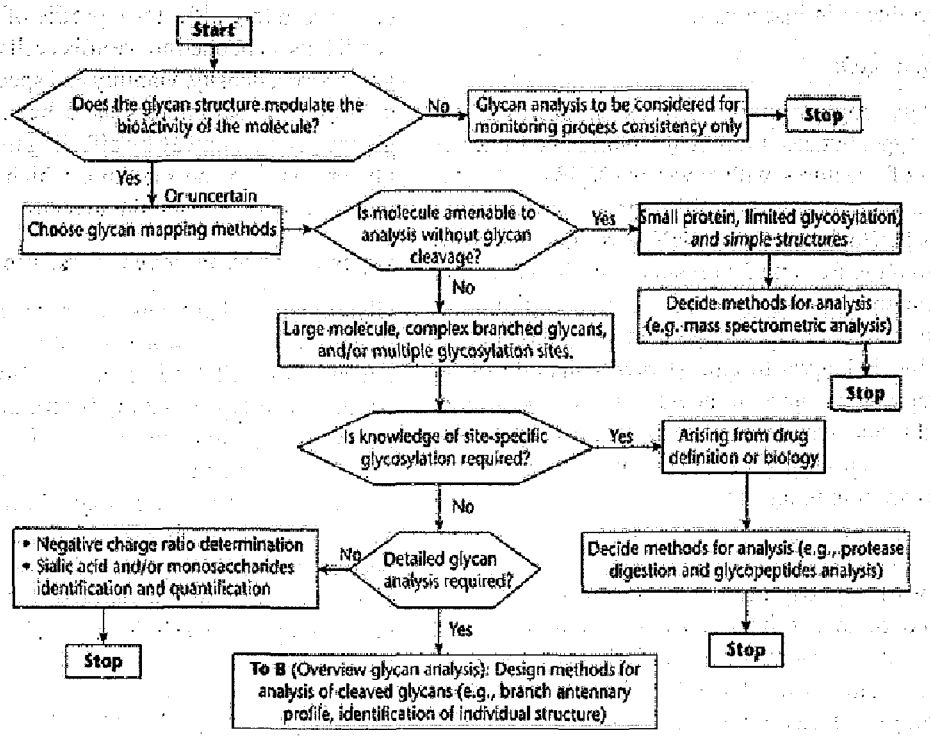

Start
Does the glycan structure modulate the bioactivity of the molecule?
No
Glycan analysis to be considered for monitoring process consistency only
Stop
Yes
Or uncertain
Choose glycan mapping methods
Is molecule amenable to analysis without glycan cleavage?
Yes
Small protein, limited glycosylation and simple structures
Decide methods for analysis (e.g., mass spectrometric analysis)
Stop
No
Large molecule, complex branched glycans, and/or multiple glycosylation sites.
Is knowledge of site-specific glycosylation required?
Yes
Arising from drug definition or biology
No
• Negative charge ratio determination
• Sialic acid and/or monosaccharides identification and quantification
No
Detailed glycan analysis required?
Decide methods for analysis (e.g., protease digestion and glycopeptides analysis)
Stop
Yes
Stop
To B (Overview glycan analysis): Design methods for analysis of cleaved glycans (e.g., branch antennary profile, identification of individual structure)

Fig. 2.2.21-3 Flow chart assists in the choice of applications for glycan analysis

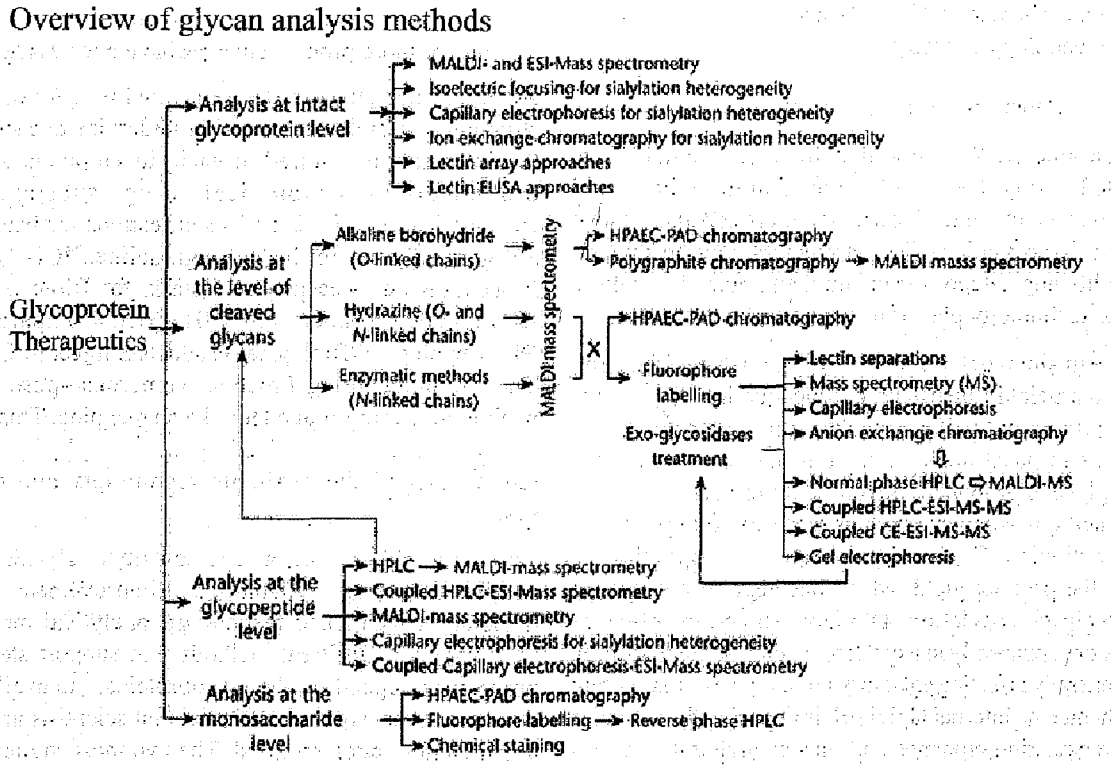


Fig. 2.2.21-4 Provides an overview of available analytical techniques and equipment employed

Glycan analysis can serve in different applications; the most important are general product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The selection of the analytical techniques and their applications in product development and routine manufacturing depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design of the strategy for manufacturing process control. For example, even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

The Fig. 2.2.21-3 flow chart assists in the choice of applications for glycan analysis, and Fig. 2.2.21-4 Provides an overview of available analytical techniques and equipment employed.

Choice of glycan analysis for characterization and specification of glycosylated biological drugs

Analysis of Intact Glycoprotein

The most direct mode of analysis is direct study of the intact molecule. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors defining biological activity is the degree of sialylation, which often determines the half-life of glycoproteins in circulation. This makes ionic-charge–based electrophoresis and ion-exchange chromatography obvious choices of technique. Nearly all types of gel electrophoresis have been used to probe protein glycosylation, including Polyacrylamide Gel Electrophoresis (PAGE), and Isoelectric Focusing (IEF). Similarly, Capillary Electrophoresis (CE), has also been found suitable. Strong anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct Mass Spectrometry (MS) is another option for the analysis of posttranslational modification. Along with ongoing improvements in the resolution of MS, more and more complex glycoproteins become accessible for direct characterization by this method.

Analysis of glycopeptides

Analysis of glycopeptides provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. Site-specific glycosylation can be affected by cell culture process conditions. Therefore, if a known glycosylation site is critical, manufacturers must monitor site-specific glycan structures. The typical approach is first to generate glycopeptides by protease digestion and to separate them by, for example, RP (reversed-phase) HPLC. Subsequently the separated glycopeptides can be further characterized individually by, e.g., direct analysis using MS, or deglycosylation and subsequent glycan profiling, as described below in the section *Profiling of cleaved oligosaccharides*.

Direct identification of the mixture of glycopeptides and nonglycosylated peptides by MS is limited by masking effects (ion suppression) of peptide signals on glycopeptide signals. One approach to overcoming this effect is to separate peptides and glycopeptides before analysis by MS, e.g., by offline coupling (matrix-assisted laser desorption ionization [MALDI]) or online coupling (electrospray ionization [ESI]). MS analysis of glycopeptides plays an important role in the characterization of O-glycans because these glycans are not always released quantitatively and because, as a result of their smaller size, they are more amenable to characterization by MS as glycopeptides. The use of CE for high-resolution separation may also be appropriate, especially for the analysis of sialylation.

Profiling of cleaved oligosaccharides

Profiling of total glycans cleaved from glycoprotein is the most common approach for the characterization of glycoproteins. It provides a way to obtain information about the various populations of glycans present on the protein. The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labelling may be needed to allow the detection of the glycans. Many protocols are available, and most of the steps in the analysis are well established. The possible drawback with such flexibility is the lack of consensus about which methods to choose under which circumstances; because of the variety of analytical techniques, comparison of results obtained by different platforms may not always be possible. So far, the majority of the work has been done on N-glycosylation, because of the following factors:

1. N-glycans usually are more clinically relevant in biologicals than O-glycans; or
2. The release of N-glycans, either by chemical means (hydrazine) or by enzymes (endoglycosidases and peptide N-glycosidase [PNGase] F), is more straightforward than is the release of O-glycans.

Deglycosylation

The approach used for the release of glycans depends on the glycoprotein under test. The cleavage agent is chosen according to the type of cleavage needed and the level of information required. Enzymatic or chemical cleavage may be used. Table 1. gives a non-exhaustive list of enzymatic cleavage agents and their specificity. Digestion efficiency generally depends on the accessibility of the glycans on the protein, and hence the protein should be denatured to maximize glycosylation site exposure unless analysts want to

Table 1 – Examples of Enzymatic Cleavage Agents

Agent	Specificity
N-linked glycan release	
Peptide-N4-(N-acetyl-β-glucosaminyl) asparagine amidase	Hydrolysis of peptide-N4-(N-acetyl-β-glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) N-acetyl-β-d-glucosaminylamine and a peptide containing an aspartate residue
Peptide N-glycosidase F (PNGase F)	Release of N-glycan chain but no release of N-glyc an chain containing (α1,3)-linked core fucose
Peptide N-glycosidase A (PNGase A)	Release of N-glycan chain containing (α1,3)-linked core fucose
Mannosyl-glycoprotein endo-β-N-acetylglucosaminidase	Endohydrolysis of the N,N'-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the –[Man(GlcNAc)2]Asn structure
Endo-β-N-acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid, and complex oligosaccharides
Endo-β-N-acetylglucosaminidase H (endo H)	Release of high-mannose and hybrid oligosaccharides
O-linked glycan release	
Glycopeptide α-N-acetylgalactosaminidase *	Hydrolysis of terminal d-galactosyl-N-acetyl-α-d-galactosaminidic residues

**This enzyme has limited usage because of its high substrate specificity.*

distinguish between surface and buried glycans. Chemical cleavage agents can also be used, e.g., hydrazine or alkaline borohydride for β-elimination of O-linked glycans.

Chemical or enzymatic release of n-glycans — PNGase F (Flavobacteriummeningosepticum) is the enzyme of choice for the release of N-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fuc α-1,3 linked to the chitobiosyl core. N-Glycan chains having this structure can be cleaved from the glycopeptide only by the almond enzyme, PNGase A. Chemical release by anhydrous hydrazine is much less common, mainly because of the limited availability of the reagent, which is considered a hazardous chemical. In addition, hydrazinolysis produces de–N-acetylated N-glycans.

Chemical or enzymatic release of o-glycans — Currently only one enzyme, O-glycanase from Diplococcuspneumoniae, is available to release O-glycans, and this enzyme has a limited usage because of its high substrate specificity: it cleaves only Galβ1,3GalNAcα-1-Ser/Thr. In addition, no ideal chemical procedure is available, but Ser- and Thr-linked O-linked glycan can usually be released by the reductive alkali-catalyzed β-elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced as soon as they are cleaved in order to prevent formation of degradation products due to peeling. However, this reaction is not specific, and in the reaction, approximately 10 per cent to 20 per cent of N-glycans are generally known to be released as well. The released glycans lack a reducing group used for the attachment of fluorescent labels by reductive amination. Fortunately, with advances in sensitive MS, direct identification of reduced glycans is possible. Relatively good quality reducing O-glycans can be obtained by alkali-catalyzed β-elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce peeled degradation products. Furthermore, O-glycan release by ethylamine is not quantitative. Hydrazine, although it may be better for use than ethylamine, requires strict control of reaction conditions and handling.

Separation of Cleaved Glycans without Fluorescent Labelling — N-Glycans can also be resolved by HPAEC high-pH anion-exchange chromatography with pulsed amperometric detection, which shows high sensitivity, can also separate some isomers, and affords the ability to directly detect native glycans without labels or tags. However, LC/MS for this separation approach is challenging because this HPAEC system uses high-pH and high-salt mobile phases that interfere with ionization of glycans. In addition, absolute quantification of the glycan is only possible if the individual PAD response factors for the different glycan structures are known, e.g., if

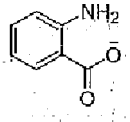
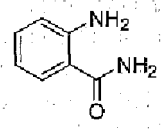
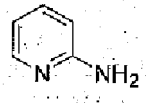
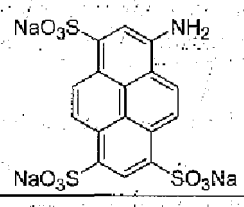
an appropriate oligosaccharide reference library is available. Porous graphitic carbon (PGC) chromatography can also be used to separate glycans, and this method adds an orthogonal selectivity compared to other columns. A PGC-electrosprayionization-MS approach also can be applied for direct glycan analysis.

MALDI/ESI-MS is a powerful method for the analysis of glycan mixtures either in the native or derivatized form. Permethylation of released glycans is a common method for direct analysis using MALDI/ESI-MS especially for sialylated glycans.

Labeling of glycans to increase detection sensitivity and/or to modify their physicochemical properties

Chemical derivatization is the most commonly used method for labelling glycans at their reducing end by reductive amination. One fluorescent label can be attached to each mono- and oligosaccharide, which facilitates determination of molar quantities. Table 2. illustrates the most common examples of fluorescent labels and their most common uses.

Table 2 – Examples of Fluorescent Labels

Name	Acronym	Structure	Analytical Technique
2-Aminobenzoic acid	2-AA		HPLC
2-Aminobenzamide	2-AB		HPLC MS
2-Aminopyridine	2-AP		HPLC
Trisodium 8-amino-pyrene-1,3,6-trisulfonic salt	APTS		CE

N-Glycan Profiling

Released glycans can be analyzed or profiled by chromatographic, electrophoretic, or MS procedures and, in general, by a combination of these. The choice of method can be grouped according to the nature of the glycans and level of information required. Analysis of glycans provides information about the various populations of glycans present on the protein (high-mannose, hybrid, complex).

Profiling of glycans by HPLC and/or by electrophoresis and MS — Profiling of fluorescent-tag–labelled glycans by HPLC has become the most common approach. One label can be attached to every single mono- and oligosaccharide by reductive amination at their reducing end, which facilitates determination of molar quantities. With the appropriate label, glycans can be profiled with high sensitivity using reversed-phase, normal-phase, and anion-exchange HPLC. Routinely, analysts use a combination of these methods in order to increase separation resolution and to better differentiate glycan structures. The accuracy of the glycan identification can be validated by means of glycan standards and/or by coupling the HPLC system with MS. Thus, anion-exchange, normal-phase, and reversed-phase HPLC–ESI–MS–MS form powerful combinations; and in-line analysis, if possible, may provide both relative quantitative profiling and information on glycan structure in a single run. Peak identification through retention time is acceptable if their identities have been previously validated by complementary methods and peak homogeneity can be assured.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecule in vivo. Anion-exchange HPLC is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. Desalting of each fraction is required before MS if the ionization interface is designed for low-salt-containing sample flows only.

High-resolution separation systems such as CE have been used to identify glycan structures without MS when well characterized standards are used for comparison. The development of an online CE–MS system has further increased the power of glycan analysis using this approach.

Structural identification by micro-enzyme sequencing and mass spectrometry — Absolute structural identification of glycans is typically obtained during development and is not necessarily the purpose of routine analysis. Traditionally, when detailed structural information is required, the analysis is usually performed using micro-enzyme sequencing. This procedure is highly dependent on the specificity and quality of the enzymes used. Combinations of enzyme sequencing, use of glycan standards and HPLC analysis can aid the structural identification. Further, the use of calibration standards, exoglycosidase reactions and suitable database searches aid in correlating the determined retention time to the glycan structure. Recently, tandem MS has been used more regularly to confirm, determine, and sequence known and novel glycan structures; this method is feasible especially when glycans are released from well-known glycoproteins and production sources.

Monosaccharide analysis

Different quantitative monosaccharide assays are carried out for a number of purposes. In the glycoprotein field, they

provide information about the relative amounts of saccharide in a glycoprotein and about the degree of sialylation of a glycoprotein; and by the measurement of monosaccharide composition, they provide some information about the structure of the glycan chains present.

The simplest assays used are colorimetric tests to demonstrate that the product is glycosylated and to quantify the total amount of saccharide present in the product. These have poor specificity between different types of sugar residues.

Assays of monosaccharide composition are generally simpler to perform than is oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content, because loss of sialylation and exposure of terminal Gal residues may lead to faster clearance of the glycoprotein from the circulation.

The assays can be divided into two types: (1) those that provide compositional information about the intact sample without prior degradation; and (2) others, principally chromatographic, that require hydrolysis of the saccharide chains before analysis and generate quantitative information about several different monosaccharide species simultaneously. In general, the former are colorimetric and the latter are chromatographic. The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of O-linked glycan chains, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GlcNAc residues is required in order to distinguish between large numbers of structurally diverse glycans. The monosaccharide N-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an unwelcome and potentially immunogenic component of biopharmaceutical products.

Sample Preparation

Glycoprotein samples for monosaccharide analysis should be free of salts, excipients, and other carrier materials (low molecular weight sugars are often used as excipients for biopharmaceuticals). This can be achieved by a number of methods, including the following:

1. Dialysis against water or a volatile buffer, using an appropriate membrane, and lyophilization;
2. HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index, and followed by lyophilization of the sample; or
3. Sample trapping on a conventional RP-SPE cartridge such as a C18 or C8 SPE system, followed by washing away of salts and excipients and elution of the required sample.

Quantification

The common method for quantification of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein–phenol mixture in water. Rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained by the simultaneous analysis of standards to generate a standard curve of absorbance against amount of saccharide and/or against a reference sample of the product under analysis.

Hydrolysis procedures for polysaccharides and glycoprotein glycan chains

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample before analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides, and residual salts may interfere with the hydrolysis or the subsequent chromatographic separation or with fluorophorelabeling. Sialic acid residues can be released either by mild acid hydrolysis or by enzymatic treatment, which leaves other sugar residues attached to the peptide backbone. Quantification of the amount of saccharide present is based on addition of an internal standard before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-d-glycero-d-galacto-2-nonulosonic acid (KDN), and 2-deoxyglucose is widely used for neutral sugars. Both of these sugars are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of the monosaccharide products during hydrolysis.

Determination of total sialic acids

Sialic acids occur in bacterial polysaccharides and glycoproteins generally as N-acetyl and N-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides by a procedure that includes acid hydrolysis to liberate constituent monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can be determined by colorimetric procedures without the need for hydrolysis. One method, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid with the product of periodate oxidation of neuraminic acid released in situ from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic

acid. For accurate quantification, include a reference standard sample is included in each measurement.

Selective release of sialic acids — Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycan chains for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required in order to release neutral and amino sugars before chromatographic analysis. The protocol must be optimized with respect to yield and saccharide degradation for each protein to be analyzed.

Neuraminidase digestion for the release of sialic acid from intact glycoproteins— Several types of neuraminidases have been isolated and studied; the enzyme derived from Clostridium perfringens is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Recombinant enzyme is available from commercial suppliers. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Acα2→,3-linked and Neu5Acα2→,6- from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known quantity of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

Separation and quantitation of unlabeled monosaccharides

Essentially the only method used for the simultaneous identification and quantification of unlabeled monosaccharide in hydrolysates is HPAEC-PAD. HPAEC-PAD methods are also applicable to oligosaccharide separations, and a single instrumental approach can be used for both applications.

HPAEC-PAD facilitates analysis of monosaccharides and all classes of oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pKa values of 12–14, and at high pH even neutral carbohydrates are ionized and can be separated as oxyanions by ion-exchange chromatography. Although separations can be performed on alkali-stable porous polystyrene–divinylbenzene anion exchangers, carbohydrates tend to exhibit broad peaks as a result of mass transfer problems. In microbeadpellicular anion-exchange column packings, small functionalized latex beads (<0.1-µm diameter) are attached to larger (<10-µm diameter) uniform nonporous beads. The carbohydrate analyte interacts with the functional groups at the surface of the latex microbeads, eliminating diffusion into and out of pores and the associated peak broadening.

PAD is the method of choice for the detection of carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. Amperometric detection measures the current, or charge, resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. Electrons are transferred from the electroactiveanalyte to the electrode during oxidation reactions and in the opposite direction during reduction reactions. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, but interfering species that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclical pulsing between high positive and negative potentials. This timed series of different potentials is referred to as a waveform, and repeated application of a waveform is the basis of pulsed amperometry. Different waveforms are used for different HPAEC-PAD applications and for different working electrodes: disposable gold electrodes require the use of fast, quadruple waveforms, but other gold electrodes allow a wider range of waveforms to be used without damaging the electrode surface. Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode recess on the sensitivity and precision of quantitative monosaccharide applications.

Fluorophore labeling of monosaccharides before separation and quantification

An alternative approach to the identification and quantification of monosaccharides present in a hydrolysate is to modify the monosaccharides by reductive amination with an easily detected fluorophore label that allows high-sensitivity detection and improves the chromatographic separation of monosaccharides. Essentially standard HPLC equipment can be used and, because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. Fluorophorelabeling has been much less widely used than HPAEC-PAD for monosaccharide identification and quantification. Labeling of sialic acid derivatives is usually undertaken with 1,2-phenylenediamine (or DMB, the 4,5-methylenedioxy derivatives), and the resulting products are separated on a C-18 column and using fluorescence detection.

Conclusion

Because of the complexity of glycoprotein glycan structures and their inherent variation during production processes, manufacturers are generally required by means of characterization studies to develop criteria for the control of

the glycosylation pattern of a biological drug substance when glycosylation occurs, as well as to develop the level of information required at each stage of production and at batch release. Then analytical procedures can be derived in a manner that provides information relevant to fulfilling quality requirements. In general, a combination of approaches and techniques is needed, and more detailed glycan structural analysis at early drug development stages is required. Validation considerations are central as method development and product knowledge progress.

Additional Definitions

High mannose—Glycan chains containing two core GlcNAc residues and between five and nine Man residues, and lacking Gal, GlcNAc, or Neu5Ac residues in the antennae. Such chains are typically found in mammalian glycans.

Hypermannosylation—(i) Addition of Man residues to high mannose chains creating chains with large numbers of Man residues, and (ii) O-Man linked glycan chains with multiple Man residues synthesized by yeast.

Paucimannose—Glycan chains containing two core GlcNAc residues between two and four Man residues. Core-linked Fucα1,3 and/or Fucα1,6 residues may be present.

Oligomannose—Used here as a generic term to include high mannose, paucimannose, and N-linked hyper-mannosylated chains.

Appendix 1

Abbreviations	
Fuc	l-Fucose
Gal	d-Galactose
GalNAc	N-Acetyl-d-galactosamine
Glc	d-Glucose
GlcNAc	N-Acetyl-d-glucosamine
Man	d-Mannose
Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid
Xyl	d-Xylose

2.2.22. Drug Substances Manufactured by Cell Culture/Fermentation

This chapter provides general requirements for the development and manufacture of products of fermentation.

These requirements are not necessarily comprehensive for all types of products manufactured and additional requirements to those prescribed in this chapter may be necessary as recommended by individual monograph or by the respective competent authority.

General

This section addresses specific controls for drug substances manufactured by cell culture or fermentation using natural or recombinant organisms.

It is not a stand alone section. In general, the principles of Good Manufacturing Practices apply to both, fermentation by classical processes and processes using recombinant and non-recombinant organisms for production of proteins and/or polypeptides, although the degree of control will differ.

In general, the degree of control for biotechnological processes used to produce proteins and polypeptides is greater than that for classical fermentation process.

The drug substances produced by biotechnological process normally consist of high molecular weight substances, such as proteins and polypeptides.

Certain drug substances with low molecular weight can also be produced by recombinant DNA technology like antibiotics, amino acids, vitamins and carbohydrates.

The level of control for these types of substances is similar to that employed for classical fermentation.

Classical fermentation is the process that use microorganisms existing in nature and/or modified by conventional methods like irradiation or chemical mutagenesis to produce drug substance.

Drug substances produced by classical fermentation are normally low molecular weight products such as antibiotics, amino acids, vitamins and carbohydrates.

Production of drug substances by fermentation involves biological processes such as inoculums development, germination or pre-fermentation process, fermentation and subsequent down stream processing for extracting, crystallizing and purification of the drug substance produced during the fermentation.

The processing steps after fermentation depend upon the mechanism of production like intracellular or extra cellular in nature.

The medium ingredients used provide nutritional potential for the optimum growth of producer organisms.

At various stages of manufacturing critical controls are necessary for bio-burden, viral contamination and/or endotoxins to produce the desired quality of drug substance for its intended purpose.

Equipments used for the manufacturing should be of desired specification and quality to facilitate the biological activities and providing necessary aeration, agitation and other critical physical parameters.

During the appropriate stages of manufacturing suitable environmental controls are necessary to minimize the risk of

contamination depending upon the type of production conditions like open, closed or contained system.

Maintenance of Cell Banks

The history of the microorganism used in the production should be documented.

The microorganism should be adequately characterized that includes phenotypic and genotypic characteristics of the microorganism.

The Master Cell Bank is a homogeneous suspension or a lyophilized content of the original cell material distributed in to individual containers for storage.

Access to cell bank should be limited to authorized personnel.

It should be stored in a fashion to maintain the viability of the cultures and prevent contamination.

Records of the storage and use of each cell bank should be maintained.

Periodically each cell bank should be monitored for its viability and suitability for use.

Cell Culture/Fermentation and In- Process Controls

Closed and contained systems are necessary to be used when the cell substrate, media, buffers and gasses are to be introduced in an aseptic manner.

Strict aseptic practices are to be followed by the personnel handling and manipulating the cultures and they should be trained scientifically.

There are several critical process parameters, which are to be monitored during the fermentation like temperature, pH, rate of aeration, rate of agitation, pressure and addition of gasses based on the process development experiments and subsequent establishment of acceptance criteria during process validation for each parameter.

Validated procedures should be available for the detection of contaminations with clearly defined instructions regarding actions to be taken if contamination is detected.

Contamination by foreign micro-organism should be isolated and identified to evaluate the possible source of contamination and its impact on the quality of final drug substance depending upon the nature and type of contamination.

Records of contamination found and subsequent handling should be maintained.

Change Control

If the production process is altered in a way that causes a significant change in the impurity profile of the drug substance, it should be addressed through change control procedure and the critical steps anticipated to be responsible for the change in impurity profile should be revalidated.

Revalidation should include the demonstration that new impurities produced as a result of change are adequately determined by test procedures with reasonable accuracy, precision and linearity.

If necessary, additions tests may be introduced with appropriate limits.

If the change in the process or microorganism results in increase in the level of impurity already present in the drug substance, the acceptability of such impurity is to be addressed.

If the Master Cell Bank is replaced, the critical steps of the production process must be revalidated to demonstrate that no adverse change has occurred in the Quality, Safety and Efficacy of the product.

Subsequent Down-stream Processing

After the fermentation cycle is over, several types of down-stream processing steps are employed for harvesting, extracting, crystallizing and purifying the substance produced.

2.2.23. Test for Biological Reactivity, *In Vitro*

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every ml of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

There are three test methods, Method A- Agar Diffusion Test, Method B- Direct Contact Test, and Method C- the Elution Test. The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use. Materials that fail the *in vitro* tests are candidates for the *in vivo* tests described in *Test for Biological Reactivity, In Vivo* (2.2.24).

Apparatus. The apparatus for the tests includes the following:

Autoclave. Use an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below, immediately following the heating cycle.

Oven. Use an oven, preferably a mechanical convection model that will maintain operating temperatures in the range of between 50° and 70° ± 2°.

Incubator. Use an incubator capable of maintaining a temperature of 37 ± 1° and a humidified atmosphere of 5 ± 1 per cent CO_2 in air.

Extraction Containers. Use containers, such as ampoules or screw-capped culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disc 50 to75 µm in thickness. A suitable disc may be fabricated from polytef.

Preparation of Apparatus. Clean all glassware thoroughly with *chromic acid mixture*, or if necessary, with hot *nitric acid*, followed by prolonged rinsing with *sterile water for injection*. Render containers and devices used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process.

NOTE—If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

Cell Culture Preparation. Prepare subcultures of L-929 (ATCC cell line CCL-1, NCTC clone 929; or other alternative cell lines obtained from a standard repository may be used with suitable validation) mammalian fibroblast cells in 10 per cent (v/v) *Serum-Supplemented Minimum Essential Medium* through trypsinization. Remove and discard cell culture medium and briefly rinse the cell layer with 0.25 per cent (w/v) Trypsin/0.53 *mM EDTA* solution. Add further 2.0 to 3.0 ml of Trypsin/EDTA solution to tissue culture flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Add 6.0 to 8.0 ml of 10.0 per cent *Foetal Bovine Serum-Supplemented Minimum Essential Medium*, aspirate cells by gently pipetting, and add appropriate aliquots of the cell suspension to new culture vessels having a seeding density of about 10^5 cells per ml. Incubate the cultures at 37° in a humidified incubator for not less than 24 hours in a 5.0 per cent CO_2 atmosphere until a monolayer, with greater than 80.0 per cent confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform and any microbial contamination.

NOTE— To avoid clumping of cells during trypsinization do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37° to facilitate dispersal.

The reproducibility of the Biological Reactivity Tests, In Vitro depends upon obtaining uniform cell culture density and any microbial contamination.

Extraction Solvents. Use *Sodium Chloride Injection* containing 0.9 per cent w/v solution of sodium chloride in *water for injections* otherwise alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used.

NOTE—Serum supplementation is used when extraction is done at 37° for 24 hours.

Procedure

Preparation of Sample for Extracts. Prepare the sample as directed in the Procedure described under in *Test for Biological Reactivity, In Vivo* (2.2.24).

Preparation of Extracts. Prepare as directed for *Preparation of Extracts* in *Test for Biological Reactivity, In Vivo* (2.2.24) using either *Sodium Chloride Injection* containing 0.9 per cent w/v solution of sodium chloride in *water for injections* or serum-free mammalian cell culture media as *Extraction Solvents.*

NOTE—If extraction is done at 37° for 24 hours in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.

Method A-Agar Diffusion Test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to be tested are applied to a piece of filter paper.

Sample Preparation. Use extracts prepared as directed, or use portions of the test specimens having flat surfaces not less than 100 mm^2 in surface area.

Positive Control Preparation. Proceed as directed for sample preparation.

Negative Control Preparation. Proceed as directed for sample preparation.

Procedure

Using 7 ml of cell suspension prepared as directed under cell culture preparation, prepare the monolayers in plates having a 60 mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum supplemented culture medium containing not more than 2.0 per cent of agar.

NOTE—The quality of the agar must be adequate to support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.

Place the flat surfaces of sample preparation, negative control preparation, and positive control preparation or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use not more than three specimens per prepared plate. Incubate all cultures for 24 hours at 37 ± 1°, preferably in a humidified incubator containing 5 ± 1 per cent of CO_2. Examine each culture around each sample, negative control, and positive control, under a microscope, using a suitable stain, if desired.

Interpretation of Results. The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of between 0 and 4 indicated in Table 1. Measure the responses of the cell cultures to the sample preparation, the negative control preparation, and the positive control preparation. The cell culture test system is suitable if the observed responses to the negative control preparation is grade 0 (no reactivity) and to the positive control preparation is at least grade 3 (moderate). If the response to the sample preparation is not more than grade 2 (mildly reactive), the sample meets the requirements of the test. Repeat the procedure if the suitability of the system is not confirmed.

Table 1 – Reactivity Grades For Agar Diffusion Test and Direct Contact Test

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen.
1	Slight	Some malformed or degenerated cells under specimen.
2	Mild	Zone limited to area under specimen and less than 0.45 cm beyond specimen.
3	Moderate	Zone extends 0.45 to 1.0 cm beyond specimen.
4	Severe	Zone extends greater than 1.0 cm beyond specimen.

Method B- Direct Contact Test

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high density materials that could cause mechanical damage to the cells.

Sample Preparation. Use portions of the test specimen having flat surfaces not less than 100 mm^2 in surface area.

Positive Control Preparation. Proceed as directed for sample preparation.

Negative Control Preparation. Proceed as directed for sample preparation.

Procedure

Using 2 ml of cell suspension prepared as directed under cell culture preparation, prepare the monolayers in plates having a 35 mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.8 ml of fresh culture medium. Place a single sample preparation, a negative control preparation, and a positive control preparation in each of duplicate cultures. Incubate all cultures for not less than 24 hours at 37 ± 1° in a humidified incubator containing 5 ± 1 per cent of CO_2. Examine each culture around each sample, negative control, and positive control preparation, under a microscope, using a suitable stain, if desired.

Interpretation of Results. Proceed as directed for Interpretation of Results under Method A.

If the response to the sample preparation is not greater than grade 2 (mildly reactive), the sample meets the requirements of the test. Repeat the procedure if the suitability of the system is not confirmed.

Method C- Elution Test

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or non physiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose response evaluations.

Sample Preparation

Prepare as directed for *Preparation of Extracts* in *Test for Biological Reactivity, In Vivo* (2.2.24) using either *Sodium Chloride Injection* containing 0.9 per cent w/v solution of *sodium chloride* in *water for injections* or serum-free mammalian cell culture media as *Extraction Solvents*. If the size of the sample cannot be readily measured, a mass of not less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per ml of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 hours in an incubator containing 5 ± 1 per cent of CO_2. Maintain the extraction temperature at 37 ± 1°, because higher temperatures may cause denaturation of serum proteins.

Positive Control Preparation. Proceed as directed for Sample Preparation.

Negative Control Preparation. Proceed as directed for Sample Preparation.

Procedure

Using 2 ml of cell suspension prepared as directed under *Cell Culture Preparation*, prepare the monolayers in plates having

a 35 mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the sample preparation, negative control preparation, or positive control preparation. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100.0 per cent). The sodium chloride injection extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25.0 per cent extract concentration. Incubate all cultures at 37 ± 1° for 48 hours in a humidified incubator preferably containing 5 ± 1 per cent of CO_2. Examine each culture at 48 hours, under a microscope, using a suitable stain, if desired.

Interpretation of Results

Proceed as directed for *Interpretation of Results* under Method A - *Agar Diffusion Test* but use Table 2. If the response to the sample preparation is not greater than grade 2 (mildly reactive), the sample meets the requirements of the test. Repeat the procedure if the suitability of the system is not confirmed. For dose response evaluations, repeat the procedure, using quantitative dilutions of the sample extract.

Table 2 – Reactivity Grades For Elution Test

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules; no cell lysis.
1	Slight	Less than or equal to 20.0 per cent of the cells are round, loosely attached, and without intra cytoplasmic granules; occasional lysed cells are present.
2	Mild	Greater than 20.0 per cent to less than or equal to 50.0 per cent of the cells are round and devoid of intra cytoplasmic granules; no extensive cell lysis and empty areas between cells.
3	Moderate	Greater than 50.0 per cent to less than 70.0 per cent of the cell layers contain rounded cells or are lysed.
4	Severe	Nearly complete destruction of the cell layers.

2.2.24. Test for Biological Reactivity, *In Vivo*

The following tests are designed to determine the biological response of animals to plastics, elastomeric and other polymeric material with direct or indirect patient contact, or by the injection or instillation of specific extracts prepared from the material under test.

It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.2 g of plastic, 0.1 g of elastomer or other material for every ml of extraction fluid. It is also essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter.

There are three tests. The *Systemic Injection Test* and the *Intracutaneous Test* are used for elastomeric materials, especially to elastomeric closures for which the appropriate *Test for Biological Reactivity, In Vitro* (2.2.23) have indicated significant biological reactivity. These two tests are used for plastics and other polymers. The third test, the *Implantation Test*, to test the suitability of these materials intended for use in fabricating containers and accessories there to, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

These three tests are applied to materials or medical devices, if there is a need for classification of plastics and other polymers based on *in vivo* biological reactivity testing.

A sample is defined as the specimen under test or an extract prepared from such a specimen. A blank consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A negative control is a specimen that gives no reaction under the conditions of the test.

Classification of Plastics

Six Plastic Classes are defined (Table 1). This classification is based on responses to a series of in vivo tests for which extracts, materials, and routes of administration are specified. These tests are directly related to the intended end-use of the plastic articles. The choice of extractants is representative of the vehicles in preparations with which the plastics are likely to be in contact. The Table 1 classification facilitates communication among suppliers, users, and manufacturers of plastics by summarizing the tests to be performed for containers for injections and medical devices if a need for classification exists.

With the exception of the *Implantation Test*, the procedures are based on the use of extracts that, depending on the heat resistance of the material, are prepared at one of three standard temperatures: 50°, 70° and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction (e.g., IV-121°, which represents a class IV plastic extracted at 121°, or I-50°, which represents a class I plastic extracted at 50°).

Plastics may be classified as Plastic Classes I–VI only on the basis of the response criteria prescribed in Table 1. This

Table 1 – Classification of Plastics

Plastic Classes[1]						Test to be conducted			
I	II	III	IV	V	VI	Test Material	Animal	Dose	Procedure[2]
×	×	×	×	×	×	Extract of sample in sodium chloride injection	Mouse	50 ml/kg	A (IV)
×	×	×	×	×	×	Extract of sample in sodium chloride injection	Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
	×	×	×	×	×	Extract of Sample in 1 in 20 solution of ethanol in Sodium chloride injection	Mouse	50 ml/kg	A (IP)
	×	×	×	×	×	Extract of Sample in 1 in 20 solution of ethanol in Sodium chloride injection	Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
		×		×	×	Extract of Sample in Polyethylene Glycol 400	Mouse	10 g/kg	A (IP)
							Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
				×	×	Extract of Sample in Polyethylene Glycol 400	Mouse	10 g/kg	A (IP)
							Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
		×	×	×	×	Extract of Sample in Vegetable Oil	Mouse	50 ml/kg	A (IP)
							Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
			×	×	×	Extract of Sample in Vegetable Oil	Mouse	50 ml/kg	A (IP)
							Rabbit or Guinea Pig	0.2 ml/animal at each of 10 or 6 sites	B (IC)
			×		×	Implant strips of Sample	Rabbit	4 strips/animal	C
			×		×	Implant Sample	Rat	2 samples /animal	C

1 Tests required for each class are indicated by "×" in appropriate columns.

2 Legend: A (IP) - Systemic Injection Test (intraperitoneal); B (IC) - Intracutaneous Test (Intracutaneous); C -Implantation Test (intramuscular or subcutaneous implantation).

classification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. Table 1 does not apply to natural elastomers, which are to be tested in *Sodium Chloride Injection* and *Vegetable Oils* only.

The *Systemic Injection Test* and the *Intracutaneous Test* are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a sample. The *Implantation Test* is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the *Sample* itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the *Implantation Test*.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a sample prepared from a specimen preconditioned by the same processing. Factors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

Apparatus. The apparatus for the tests includes the following:

Autoclave. Use an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a

pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below, immediately following the heating cycle.

Oven. Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of 50° or 70 ± 2°.

Extraction Containers. Use containers such as ampoules or screw-capped culture test tubes, of Type I glass. If used, culture test-tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disc 0.05–0.075 mm in thickness. A suitable disc may be fabricated from a polytef resin.

Preparation of apparatus. Clean all glassware thoroughly with *chromic acid mixture*, or if necessary, with hot *nitric acid*, followed by prolonged rinsing with *water*. Clean the cutting devices by an appropriate method (e.g., successive cleaning with *acetone* and *methylene chloride*) prior to use in subdividing a specimen.

Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water. Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process.

NOTE—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.

Extracting media

(a) *Sodium Chloride Injection.* A 0.9 per cent w/v solution of *sodium chloride* in *water for injections*.

(b) A 5 per cent v/v solution of *ethanol* in *sodium chloride injection*.

(c) *Polyethylene Glycol 400.*

(d) *Vegetable Oil.* Use freshly refined sesame oil or cottonseed oil or other suitable vegetable oils that meets the following additional requirement. Using three properly prepared animals, and inject the oil 0.2 ml intracutaneously at each of 10 sites per animal, and examine the animals at 24, 48, and 72 hours following injection. Rate the observations at each site on the numerical scale indicated in Table 2. For the 3 rabbits or guinea pigs (30 or 18 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.

(e) Drug Product Vehicle (where applicable).

(f) *Water for Injection.*

Table 2– Evaluation of Skin Reactions[1]

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined Erythema	2
Moderate to severe Erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema Formation[2]	**Score**
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

[1]Draize JH, Woodward G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 1944; 82: 377–390.

[2]Excludes non-inflammatory (mechanical) edema from the blank or extraction fluid.

NOTE—Suitable validated in vitro alternative tests, procedures where such alternatives are available should be used.

Preparation of Sample. Both the *Systemic Injection Test* and the *Intracutaneous Test* may be performed using the same extract, if desired or separate extracts may be made for each test. Select and subdivide into portions a *Sample* of the size indicated in Table 3. Remove particulate matter, such as lint and free particles, by treating each subdivided sample or negative control as follows.

Place the sample into a clean, glass-stopper, 100 ml graduated cylinder of Type I glass and add about 70 ml of *water for injection*. Agitate for about 30 seconds, and drain off the water. Repeat this step, and dry those pieces prepared for the extraction with *vegetable oil* in an oven at a temperature not exceeding 50°.

NOTE—Do not clean the Sample with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.

Preparation of Extracts. Place a properly prepared sample under examination in an extraction container and add 20 ml of the appropriate extracting medium. Repeat these directions for each extracting medium required for testing. Also, prepare one 20 ml blank of each medium for parallel injections and

Table 3 – Surface Area of Specimen to be Used[1].

Form of Material	Thickness	Amount of sample for each 20 ml of Extracting Medium (both sides combined)	Subdivided into
Film or sheet	Less than 0.5 mm	Equivalent of 120 cm^2 total surface area	Strips of about 5 × 0.3 cm
	0.5 to 1 mm	Equivalent of 60 cm^2 total surface area (both sides combined)	
Tubing	Less than 0.5 mm (wall)	Length (in cm) = 120 cm^2/(sum of ID and OD circumferences)	Sections of about 5 × 0.3 cm
	0.5 to 1 mm (wall)	Length (in cm) = 60 cm^2/(sum of ID and OD circumferences)	
Slabs, tubing, and molded items	More than 1 mm	Equivalent of 60 cm^2 total surface area (all exposed surfaces combined)	Pieces up to about 5 × 0.3 cm
Elastomers	More than 1 mm	Equivalent of 25 cm^2 total surface area (all exposed surfaces combined)	Do not Subdivide[2]

[1]When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 ml of extracting fluid.

[2]Molded elastomeric closures are tested intact.

comparisons. Extract by heating in an autoclave at 121° for 60 minutes, in an oven at 70° for 24 hours, or at 50° for 72 hours. Allow adequate time for the liquid within the container to reach the extraction temperature.

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature between 20° and 30°, and do not use for tests after 24 hours. Of importance are the contacts of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

NOTE—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the Sample pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with vegetable oil, seal the screw caps adequately with pressure-sensitive tape.

Systemic Injection Test. This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice. Alternate routes of injection may be used with justification.

Test Animals. Use healthy, not previously used albino mice weighing between 17 and 23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, *ad libitum*.

Procedure

NOTE— Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.

Inject each of the five mice in a test group with the sample or the blank as outlined in Table 4, except to dilute each g of the extract of the sample prepared with *Polyethylene glycol 400*, and the corresponding blank with 4.1 volumes of sodium chloride injection to obtain a solution having a concentration of about 200 mg of *polyethylene glycol* per ml.

Observe the animals immediately after injection, again 4 hours after injection, and then at least at 24, 48 and 72 hours. If during the observation period none of the animals treated with the extract of the sample shows a significantly greater biological reactivity than the animals treated with the blank, the sample meets the requirements of this test. If two or more mice die, or if abnormal behaviour such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 2 g occurs in three or more mice, the sample does not meet the requirements of the test. If any animals treated with the sample show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. In the repeat test, all 10 animals treated with the sample show no significant biological reactivity above the animals tested with blank during the observation period.

Table 4 – Injection Procedure- Systemic Injection Test

Extract or Blank	Dose per kg	Route[1]
Sodium Chloride Injection	50 ml	IV
5 per cent v/v solution of Ethanol in Sodium Chloride Injection	50 ml	IV
Polyethylene Glycol 400	10 g	IP
Drug product vehicle (where applicable)	50 ml	IV
	50 ml	IP
Vegetable Oil	50 ml	IP

[1]IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Intracutaneous Test. This test is designed to evaluate local response to the extracts of materials being examined following intracutaneous injection into rabbits or guinea pigs.

Test Animals. Select healthy, rabbits or guinea pigs with fur that can be clipped closely and skin that is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue.

Procedure

NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.

On the day of the test, closely clip the fur on the animal's back on both side of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted *ethanol*, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit or guinea pig, if it is determined that the test results will not be affected. For each sample use two animals and inject each intracutaneously, using one side of the animal for the sample and the other side for the blank, as outlined in Table 5.

NOTE—Dilute each g of the extract of the sample prepared with Polyethylene Glycol 400, and the corresponding blank, with 7.4 volumes of sodium chloride injection to obtain a solution having a concentration of about 120 mg of polyethylene glycol per ml.

Table 5 – Intracutaneous Test

Extract or Blank	Number of Sites (per animal)	Dose (µl per site)
Sample	5	200
Blank	5	200

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted *ethanol* to facilitate reading of injection sites. Observe all animals at 24, 48 and 72 hours after injection. Rate the observations on a numerical scale for the extract of the sample and for the blank, using Table 2. Reclip the fur as necessary during the observation period. The average erythema and edema scores for sample and blank sites are determined at every scoring interval (24, 48 and 72 hours) for each rabbit or guinea pig. After the 72 hour scoring, all erythema scores plus edema scores are totalled separately for each sample and blank.

Divide each of the totals by 12 (2 animals x 3 scoring periods x 2 scoring categories) to determine the overall mean score for each sample versus each corresponding blank. The requirements of the test are met if the difference between the sample and the blank mean score is 1.0 or less. If at any observation period the average reaction to the sample is questionably greater than the average reaction to the blank, repeat the test using three additional rabbits or guinea pigs. The requirements of the test are met if the difference between the sample and the blank mean score is 1.0 or less.

Implantation Test. The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. The intramuscular implantation test requires healthy adult albino rabbits. The test specimens are placed into needles as the delivery system for implantation.

Although most materials lend themselves readily to this method, there are a number of materials that are unsuitable for intramuscular implantation. For materials with physical characteristics unsuitable for routine intramuscular implantation, the subcutaneous rat implantation model is a viable alternative.

Intramuscular Implantation in Rabbits. Prepare for implantation 8 strips of the sample and 4 strips of *High-Density Polyethylene RS*. Each strip should measure not less than 10 x 1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15–19 gauge) with intravenous point and a sterile trocar. Use either presterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure.

NOTE—Allow for proper degassing if agents such as ethylene oxide are used.

Test Animals. Select healthy, adult rabbits weighing not less than 2.5 kg, and with paravertebral muscles that are sufficiently

large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the para vertebral site. The animals must be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching. CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines for time being in force should be used for conducting animal experiments.

Procedure

Perform the test in a clean area. On the day of the test or up to 20 hours before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted *ethanol*, and dry the skin prior to injection.

Implant four strips of the sample into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5–5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of *High-Density Polyethylene RS* in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another strip.

Keep the animals for a period of not less than 120 hours, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens and auxiliary light source. Observe the sample and control implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. If present, measure encapsulation, by recording the width of the capsule (from the periphery of the space occupied by the implant control or sample to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to Table 6.

Table 6 – Evaluation of Encapsulation in the Implantation Test

Capsule width	Score
None	0
Up to 0.5 mm	1
0.6–1.0 mm	2
1.1–2.0 mm	3
Greater than 2.0 mm	4

Calculate the differences between average scores for the sample and control sites. The requirements of the test are met if the difference does not exceed 1.0, or if the difference between the sample and control mean scores for more than one of the four implant sites does not exceed 1 for any implanted animal.

Subcutaneous Implantation in Rats. Prepare for implantation 10 sample specimens and 10 control specimens. The size and shape of the control specimens shall be as similar to that of the test specimens as practically possible. For example, specimens made of sheeting material shall be 10–12mm in diameter and from 0.3–1mm in thickness. The edges of the specimens should be as smooth as possible to avoid additional mechanical trauma upon implantation.

Test Animals. Select healthy albino rats weighing between 225 to 350 g at the time of implantation.

Procedure

NOTE—Perform the experiment as per CPCSEA guidelines.

Perform the test in a clean area. Anesthetize for times being in force the animal until a surgical plane is achieved. Clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Clean the clipped area with povidone–iodine solution. Make two midline incisions (approximately 1.0 cm long) through the skin at the cranial and caudal regions on the dorsal surface by using aseptic technique. Using blunt dissection, separate the fascia connecting skin to muscle to form a pocket underneath the skin lateral to each side of the incision (base of pocket approximately 20 mm from the line of implant). Insert a sterile sample into each pocket, and close the incision with wound clips or sutures. Implant two test samples and two control samples in each of five rats. Keep the animals for a period of at least seven days, and sacrifice them at the end of the observation period by CO_2 induced hypoxia or administering an overdose of an anesthetic agent. Allow sufficient time to elapse for the tissue to be cut without bleeding. Cut the skin (dorsal surface) longitudinally and lay back. Carefully examine macroscopically the area of the tissue surrounding the implant. Cut the sample in half and remove for close examination of the tissue in direct contact with the sample. Use a magnifying lens and auxiliary light source, if appropriate.

Observe the sample and control implant sites for hemorrhage, necrosis, discolorations, and infections and record the observations. If present, measure encapsulation by recording the width of the capsule (from the periphery of the space occupied by the implant control or sample to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to Table 6. Calculate the differences between average scores for the sample and control sites. The requirements of the test are met if the difference does not exceed 1.0.

Abnormal Toxicity (2.2.1). Complies with the test for abnormal toxicity using General test and observe the mice for 48 hours.

2.2.25. Monocyte Activation Test

The monocyte activation test (MAT) is a test that is based on the reaction of human blood cells [monocytes] to pyrogenic substances. The monocytes while reacting to the pyrogenic substances produce cytokines such as Interleukin [IL – 6], Tumour Necrosis Factor-α [TNFα] etc. This is a part of the fever response in humans. The MAT assay measures the cytokines produced during the reaction with pyrogens and therefore can co-relate to the pyrogenicity of the product. Similarly the temperature increase measured in the Rabbit Pyrogen Test is correlated to the pyrogenicity of the product. Therefore, the MAT test, upon due validation, can be used as a suitable alternate to the Rabbit Pyrogen Test.

In order to use the monocyte activation test as an alternative to rabbit pyrogen test, validation data for the applicability of the MAT to the product is required. Validation data from 3 production batches should include details of sample preparation to adequately address the interfering factors. Additionally, the data from 3 production batches to correlate the rabbit pyrogen test with MAT needs to be provided to establish equivalence.

In the absence of a validated non-endotoxin standard and since the pyrogens group of substances includes bacterial endotoxins, an endotoxin reference standard that has been calibrated against the International Endotoxin Standard could be used for the MAT.

Additionally, as Bacterial Endotoxin Test (BET) specifically detects bacterial endotoxins and a good correlation has been established between the bacterial endotoxins and the rabbit pyrogenic response, we would need to use the bacterial endotoxin standards and follow the related established safety limits such as threshold endotoxin concentration and allowable endotoxin concentration while using the MAT.

The following two methods could be used for carrying out MAT test.

1. Quantitative test
2. Semi-quantitative test

The test should be carried out in a manner that avoids pyrogen contamination.

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the pyrogen limit can be determined. Determine the MVD using the following expression:

$$\frac{PL \times C}{LOD}$$

where, PL = pyrogen limit;
C = concentration of test solution;
LOD = limit of detection.

The acceptance criterion for a pass/fail is the pyrogen limit (PL), which is expressed in endotoxin equivalents per milligram or millilitre, or in units of biological activity of the preparation under examination. The PL is calculated using the following expression.

$$\frac{K}{M}$$

where, K = threshold pyrogenic dose of endotoxin per kilogram of body mass;
M = maximum recommended bolus dose of product per kilogram of body mass.

The endotoxin limits depends on the product and its route of administration and is stated in monographs.

Values for K suggested are:

Route of administration	K (EU of endotoxin per kilogram of body mass)
Intravenous	5.0
Intravenous, for radiopharmaceuticals	2.5
Intrathecal	0.2

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

Where an endotoxin limit (EL) has been specified for a product, the PL is the same as the EL, unless otherwise prescribed. In this case, the concentration of test solution is expressed in mg/ml if the endotoxin limit is specified by mass (EU/mg), in Units/ml if the endotoxin limit is specified by unit of biological activity (EU/Unit), in ml/ml if the endotoxin limit is specified by volume (EU/ml).

The cut-off value is calculated using the following expression:

$$\bar{n} + 3\sigma$$

where, $\bar{n}$ = mean of the 4 replicates for the responses to the blank A_e.
σ = standard deviation of the 4 replicates of the responses to the blank A_e.

The cut-off value is expressed in units appropriate to the read-out.

The limit of detection (LOD) is determined using the endotoxin standard curve. The LOD is the concentration of endotoxin corresponding to the cut-off value. For the purpose of the test, the LOD is expressed as endotoxin equivalents per ml.

General Procedure

A test solution of the sample is incubated with a source of human monocytes or human monocytic cells and the responses viz. levels of cytokines synthesised are compared with responses to standard endotoxin being tested. The chosen read-out method is calibrated using the appropriate standard.

The source of human monocytes or human monocyte cells can be from human heparinised peripheral blood that is not more than 4 hours old, or human peripheral blood mononuclear cells (PBMC) isolated, and diluted with appropriate medium or saline to 2 to 50 per cent v/v (final concentration) so as to achieve a final cell density of 0.1 to 1.0×10^6 cells per receptacle.

Cell Sources and Qualification

Whole Blood

The whole blood needs to be obtained from single donor or from pooled whole blood. It should be in line with the requirements described under Qualification of blood donors and Qualification of cells pooled from a number of donors respectively.

Peripheral Blood Mononuclear Cells (PBMC)

PBMC are isolated from blood obtained from single donors or from pooled whole blood. The cells should be in line with the requirements described under Qualification of blood donors and Qualification of cells pooled from a number of donors respectively.

Qualification of Blood Donors

Blood donors are to satisfy the following qualification criteria:

1. Donor should be in good health.
2. Donor should not be suffering from any bacterial or viral infections.
3. Donor should be free from the symptoms of any infection for a period of at least one week prior to the donation of blood.
4. Donor is not to have taken non-steroidal anti-inflammatory drugs during the 48 hours prior to donating blood
5. Donor is not on steroidal anti-inflammatory drugs during the 7 days prior to donating blood.

Following cases would not qualify as blood donors:

i. Individuals who have been administered immune-suppressants.

ii. Individuals who have been administered drugs known to influence the production of the chosen readout.

Qualification of Cells Pooled From a Number of Donors

Pools (of whole blood or blood fractions, e.g., PBMC), must be from a minimum of 4 individual donors, preferably equal volumes of blood or cells from each of them. For the qualification of pooled cells, within 4 hours of collection of blood, a dose-response curve should be generated using standard endotoxin concentrations.

Preparatory Testing

The manufacturer's instructions of the MAT kits need to be followed to establish the appropriate standard curve, determine the non-interfering product concentration so as to ensure detection of the pyrogens.

Test for Interfering Factors

Initial test must be performed to ensure that the test solution does not have interfering factors. It is necessary to re-validate the test method when any changes are effected to the test procedure that are likely to influence the result of the test. Prepare dilutions not exceeding MVD of the product and spike with endotoxin concentration near or equal to the mid-point of the standard curve or equal to twice the LOD. The mean recovery needs to be calculated by subtracting the mean concentration of endotoxin equivalents in the solution (if any) from that in the spiked solution. The test solution is considered free of interfering factors if the recovery of endotoxin is within 50 to 200 per cent of the spiked endotoxin concentration.

Interpretation of the test results

The test samples complies with the test when:

1. All negative controls are negative.
2. Spike recovery is between 50 per cent and 200 per cent.
3. The pyrogen concentration of the test solution is less than the pyrogen limit.

2.2.26. Microbiological Assay of Calcium Pantothenate

The microbiological assay of a calcium pantothenate is based upon a comparison of the growth of microorganism by measured concentrations of the calcium pantothenate under examination that produced by known concentrations of a standard preparation of the calcium pantothenate having a known activity.

Generally turbidimetric (or tube assay) method is usually employed which depends upon the growth of a microbial culture in a uniform solution of the calcium pantothenate in a fluid medium that is favourable to its rapid growth in the presence of calcium pantothenate.

Media. Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 1. Minor modifications of the individual ingredients may be made, or

reconstituted dehydrated media may be used provided the resulting media have equal or better growth-promoting properties and give a similar standard curve response.

Table 1– Basal Media: Quantities in g per ml per 250 ml

Ingredient	Quantities
Acid-hydrolyzed Casein Solution	25 ml
Cystine-Tryptophane Solution	25 ml
Polysorbate 80 Solution	0.25 ml
Dextrose, Anhydrous	10.0 g
Sodium acetate, Anhydrous	5.0 g
Adenine-Guanine –Uracil Solution	5 ml
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 ml
Para-amino benzoic Acid-Niacin-Pyridoxine Hydrochloride Solution	5 ml
Salt Solution A	5 ml
Salt Solution B	5 ml

Dissolve the *anhydrous dextrose* and *sodium acetate* in the solutions previously mixed and add sufficient *water* to produce 250 ml and mix. Adjust the pH so that after sterilisation pH is 6.8 with sufficient *1 M sodium hydroxide* or *1 M hydrochloric acid*.

Acid-Hydrolyzed Casein Solution. Mix 100.0 g of vitamin-free casein with 500 ml of 6 *M hydrochloric acid*, and reflux the mixture for 8 to 12 hours. Remove the *hydrochloric acid* from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in *water*, adjust the solution with *1 M sodium hydroxide* to a pH of 3.5 ± 0.1, and add sufficient *water* to produce 1000 ml. Add 20.0 g of *activated charcoal*, stir for 1 hour, and filter. Repeat the treatment with *activated charcoal*. Store under *toluene*. Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution. Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of DL-tryptophane) in 700 to 800 ml of *water*, heat to 70° to 80°, and add dilute *hydrochloric acid* (1 in 2) drop wise, with stirring, until the solids are dissolved. Cool, and add sufficient *water* to produce 1000 ml. Store under *toluene*.

Adenine-Guanine-Uracil Solution. Dissolve 200 mg each of adenine sulphate, guanine hydrochloride and uracil, with the aid of heat, in 10 ml of *4 N hydrochloric acid*, cool, and add sufficient *water* to produce 200 ml. Store under *toluene* in a refrigerator.

Polysorbate 80 Solution. Dissolve 25.0 g of *polysorbate 80* in *ethanol* to produce 250 ml.

Riboflavin-Thiamine Hydrochloride-Biotin Solution. Prepare a solution containing, in each ml, 20 µg of *riboflavin*, 10 µg of *thiamine hydrochloride* and 0.04 µg of biotin, by dissolving *riboflavin, thiamine hydrochloride*, and biotin in *0.02 M acetic acid*. Store under *toluene* and protected from light.

Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution. Prepare a solution in neutral 25 per cent *ethanol* to contain 10 µg of *para-aminobenzoic acid*, 50 µg of *niacin*, and 40 µg of *pyridoxine hydrochloride* in each ml. Store in a refrigerator.

Salt Solution A. Dissolve 25 g of *mono basic potassium phosphate* and 25 g of *dibasic potassium phosphate* in *water* to produce 500 ml. Add 5 drops of *hydrochloric acid*, and store under *toluene*.

Salt Solution B. Dissolve 10 g of *magnesium sulphate*, 0.5 g of *sodium chloride*, 0.5 g of *ferrous sulphate*, and 0.5 g of *manganese sulphate* in sufficient *water* to produce 500 ml. Add 5 drops of *hydrochloric acid*, and store under *toluene*.

Preparation of the standard solution. To prepare a stock solution, dissolve 50 mg of the *calcium pantothenate RS*, accurately weighed, previously dried and stored in the dark over *phosphorus pentoxide* while protected from absorption of moisture during the weighing, in about 500 ml of *water* in a 1000 ml volumetric flask. Add 10 ml of *0.2 M acetic acid* and 100 ml of *sodium acetate solution* (1 in 60), then dilute with *water* to volume. Each ml represents 50 µg of *calcium pantothenate RS* and store under *toluene* in a refrigerator.

On the day of the assay, prepare from the stock solution by diluting the stock solution with sufficient *water* so that it contains between 0.01 µg and 0.04 µg of *calcium pantothenate RS* per ml. The exact concentration being such that the responses obtained as directed for Procedure 2.0 and 4.0 ml of the Standard Preparation being used are within the linear portion of the log-concentration response curve.

Preparation of the sample solution. Prepare a solution of sample under examination contain approximately the equivalent of the calcium pantothenate concentration in the standard solution.

Test Organism. *Lactobacillus plantarum*, ATCC 8014/ *Lactobacillus plantarum*, MTCC 2621/ *Lactobacillus pentosus* NCIM 2912/ *Lactobacillus plantarum* NCIB 8030. Maintain a culture on stab of the medium and under the incubation conditions, and transfer weekly to fresh stab as follows.

Dissolve 2.0 g of water-soluble yeast extract in 100 ml of *water*, add 500 mg of *anhydrous dextrose*, 500 mg of *anhydrous sodium acetate*, and 1.5 g of *agar*, and heat the mixture, with stirring, on a steam bath, until the *agar* dissolves. Add approximately 10 ml portions of the hot solution to test tubes, suitably close or cover the tubes, sterilize at 121° and allow the tubes to cool in an upright position. Prepare stab cultures

in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum/ Lactobacillus pentosus*. Incubate at 30° to 35° but held constant to within ± 0.5° for 16 to 24 hours. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, do not use for inoculum if the culture is more than 1 week old.

Culture Medium. To each of a series of test tubes containing 5.0 ml of Basal Medium Stock Solution, add 5.0 ml of *water* containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° and cool.

Preparation of inoculum. Prepare the microbial suspensions for the inoculum for the assay by transfer of cells from the stock culture of *Lactobacillus plantarum/ Lactobacillus pentosus* to a sterile tube containing 10 ml of culture medium. Incubate at 30° to 37° but held constant to within ± 0.5° for 16 to 24 hours.

Assay Design

Prepare five different concentrations in test tubes in duplicate 1.0, 2.0, 3.0, 4.0 and 5.0 ml respectively of the standard solution for preparing the standard curve by diluting the stock solution of the standard solution of the calcium pantothenate/ pantothenic acid. To each tube and to 4 similar tubes containing no standard solution add 5.0 ml of Basal Medium Stock Solution and sufficient *water* to produce 10 ml.

To similar test tubes add volumes of the test solution corresponding to 3 or more of the levels as for the standard solution, including the levels of 2.0, 3.0 and 4.0 ml in duplicate. To each tube add 5.0 ml of Basal Medium Stock Solution and sufficient *water* to produce 10 ml.

Cover all tubes suitably to prevent contamination, and heat in an autoclave at 121° for 5 minutes. Cool and add 1 drop of inoculum to each tube, except 2 of the 4 tubes containing no standard solution (to serve as the uninoculated blanks), and mix. Incubate the test tubes at 30° to 35° but held constant to within ± 0.5° for 16 to 24 hours.

Mix the content of each tube and measure the growth of the test organism by determining the transmittance of the tubes by a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube. With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Estimation of potency. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the ml of standard solution per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight response line or smooth curve that best fits the plotted points.

Calculate the response y, adding together the two transmittances for each level of the sample solution. Read from the standard curve the logarithm of the volume of the standard solution corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume in ml, of the sample solution to obtain the difference x, for each dosage level. Average the values of x for each of three or more dosage levels to obtain $\bar{x} = M'$, the log relative potency of the sample solution.

Determine the quantity, in mg, *calcium pantothenate RS* corresponding to the calcium pantothenate in the portion of material taken for assay as

$$\text{antilog}(M) = \text{antilog}(M' + \log R)$$

in which R is the number of mg of calcium pantothenate that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay.

2.2.27. Microbiological Assay of Vitamin B_{12} Activity

The microbiological assay of a vitamin B_{12} is based upon a comparison of the growth of microorganism by measured concentrations of the vitamins B_{12} under examination that produced by known concentrations of a standard preparation of the vitamin B_{12} having a known activity.

Turbidimetric (or tube assay) method is usually employed which depends upon the growth of a microbial culture in a uniform solution of the vitamin B_{12} in a fluid medium that is favourable to its rapid growth in the presence of vitamin B_{12}.

Media. Prepare the media required for the preparation of test organism inocula from the ingredients listed in Table 1 and 2. Minor modifications of the individual ingredients may be made, or reconstituted dehydrated media may be used provided the resulting media have equal or better growth-promoting properties and give a similar standard curve response.

Table 1 – Basal Media: Quantities in g per ml per 250 ml

Ingredient	Quantities
L-Cystine	0.1 g
L-Tryptophane	0.05 g
1*M Hydrochloric Acid*	10 ml
Adenine-Guanine-Uracil Solution	5 ml
Xanthine Solution	5 ml
Vitamin Solution I	10 ml
Vitamin Solution II	10 ml
Salt Solution A	5 ml
Salt Solution B	5 ml
Asparagines Solution	5 ml
Acid-hydrolyzed Casein Solution	25 ml
Dextrose, Anhydrous	10 g
Sodium acetate, Anhydrous	5 g
Ascorbic Acid	1 g
Polysorbate 80 Solution	5 ml

Add the ingredients in the table, carefully dissolving the *cystine* and *tryptophane* in the *hydrochloric acid* before adding the next eight solutions in the resulting solution. Add 100 ml of *water*, mix and dissolve the *dextrose, sodium acetate* and *ascorbic acid*. Filter if necessary, add the *polysorbate 80* solution and add sufficient *water* to produce 250 ml. Adjust the pH so that after sterilisation it is 5.5-6.0 with sufficient *1 M sodium hydroxide*.

Acid-Hydrolyzed Casein Solution. Mix 100.0 g of vitamin-free casein with 500 ml of *6 M hydrochloric acid*, and reflux the mixture for 8 to 12 hours. Remove the *hydrochloric acid* from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in *water*, adjust the pH of solution 3.5 ± 0.1 with *1 M sodium hydroxide*, and add sufficient *water* to produce 1000 ml. Add 20.0 g of *activated charcoal*, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under *toluene* in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Asparagine Solution. Dissolve 2.0 g of *L-asparagine* in *water* to produce 200 ml. Store under *toluene* in a refrigerator.

Adenine-Guanine-Uracil Solution. Dissolve 200 mg each of *adenine sulphate, guanine hydrochloride* and *uracil*, with the aid of heat, in 10 ml of *4 M hydrochloric acid*, cool, and add sufficient *water* to produce 200 ml. Store under *toluene* in a refrigerator.

Xanthine Solution. Suspend 0.20 g of *xanthine* in 30 ml to 40 ml of *water*, warm to about 70°, add 6.0 ml of *6 M ammonium hydroxide*, and stir until the solid is dissolved. Cool, and add *water* to produce 200 ml. Store under *toluene* in a refrigerator.

Salt Solution A. Dissolve 10 g of *mono basic potassium phosphate* and 10 g of *dibasic potassium phosphate* in *water* to produce 200 ml. Add 2 drops of *hydrochloric acid*, and store under *toluene*.

Salt Solution B. Dissolve 4.0 g of *magnesium sulphate*, 0.2 g of *sodium chloride*, 0.2 g of *ferrous sulphate*, and 0.2 g of *manganese sulphate* in sufficient *water* to produce 200 ml. Add 2 drops of *hydrochloric acid*, and store under *toluene*.

Polysorbate 80 Solution. Dissolve 20.0 g of *polysorbate 80* in *ethanol* to produce 200 ml. Store in a refrigerator.

Vitamin Solution I. Dissolve 10 mg of *riboflavin*, 10 mg of *thiamine hydrochloride*, 100 µg of biotin, and 20 mg of *niacin* in *0.02 M glacial acetic acid* to produce 400 ml. Store under *toluene* in a refrigerator and protected from light.

Vitamin Solution II. Dissolve 20 mg of *para-aminobenzoic acid*, 10 mg of *calcium pantothenate*, 40 mg of *pyridoxine hydrochloride*, 40 mg of *pyridoxal hydrochloride*, 8 mg of *pyridoxamine dihydrochloride*, and 2 mg of folic acid in dilute neutralized *ethanol* (1 in 4) to produce 400 ml. Store in a refrigerator and protected from light.

Tomato Juice Preparation. Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g in 1000 ml of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under *toluene* in a refrigerator.

Table 2 – Culture Media: Quantities in g per ml per 100 ml

Ingredient	Quantities
Water-soluble yeast extract	0.75 g
Dried peptone	0.75 g
Anhydrous dextrose	1.0 g
Potassium dihydrogen phosphate	0.20 g
Tomato juice Preparation	10 ml
Polysorbate 80 Solution	1 ml

Add the ingredients in the table, and dissolving in 100 ml water. Adjust the pH with sufficient *1 M sodium hydroxide* so that after sterilisation it is 6.8. Place 10 ml portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 minutes. Cool as rapidly as possible to avoid colour formation resulting from overheating the medium.

Preparation of the standard solution. To prepare a stock solution, dissolve a quantity of *Cyanocobalamin RS*, accurately weighed, add sufficient 25 per cent *ethanol* to obtain a solution having concentration of about 1.0 µg per ml and mix. Store in a refrigerator.

On the day of the assay, prepare from the stock solution by dilute the stock solution with sufficient *water* to a measured volume such that after incubation period as described under Assay Design, the transmittance between the inoculated blank and the 5.0 ml level of the standard cyanocobalamin solution is not less than that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng and 0.04 ng per ml standard solution. Prepare fresh standard solution for each assay.

Preparation of the sample solution. Prepare a solution of sample under examination contain approximately the equivalent of the Vitamin concentration B_{12} in the standard solution. Take a suitable quantity of the material to be assayed, previously reduced to a fine powder if necessary, and accurately measured or weighed, in an appropriate vessel containing, for each g or ml of material taken, 25 ml of an aqueous extracting solution (freshly prepared and each 100 ml contains 1.29 g of *disodium phosphate*, 1.1 g of *anhydrous citric acid*, and 1.0 g of *sodium metabisulphite*). Autoclave the mixture at 121° for 10 minutes. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin B_{12} activity approximately equivalent to that of the standard cyanocobalamin Solution which is added to the assay tubes.

Suspension Medium. Dilute a measured volume of Basal Medium Stock Solution with an equal volume of water. Place 10 ml portions of the diluted medium in test tubes. Sterilize, and cool as directed above for the Culture Medium.

Test Organism. *Lactobacillus leichmannii,* ATCC 7830/ *Lactobacillus leichmannii,* NCIM 2071/ *Lactobacillus leichmannii* NCTC 8118. Maintain a culture on stab of the medium and under the incubation conditions as follows.

To 100 ml of Culture Medium add 1.0 g to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Place approximately 10 ml portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121° for 15 minutes in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii.* Incubate at 30° to 40° but held constant to within ± 0.5° for 16 to 24 hours. Store at 2° to 8°.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture. A slow growing culture seldom gives a suitable response curve, and may lead to erratic results.

Preparation of inoculum. Make a transfer of cells from the Stock Culture of *Lactobacillus leichmannii* to 2 sterile tubes containing 10 ml of the Culture Medium each. Incubate these cultures at 30° to 40° but held constant to within ± 0.5° for 16 to 24 hours.

NOTE — A frozen suspension of Lactobacillus leichmannii may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.

Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 ml of sterile Suspension Medium, and combine. Using sterile Suspension Medium, adjust the volume in normal saline to produces 70 per cent transmittance when read on a spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10 mm cell, and read against normal saline set at 100 per cent transmittance. Prepare a 1 in 400 dilution of the adjusted suspension using Basal Medium Stock Solution, and use it for the test inoculum. (This dilution may be altered, when necessary, to obtain the desired test response).

Apparatus

All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organism is heated at 250° for 2 hours.

Assay Receptacles. For assay tubes, use glass or plastic test-tubes, e.g. 20 mm x 150 mm. Cleanse thoroughly to remove all residues and traces of cleaning solution and sterilise tubes that have been used previously before subsequent used because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity.

Assay Design

Place 1.0 ml, 1.5 ml, 2.0 ml, 3.0 ml, 4.0 ml, and 5.0 ml, respectively concentration of the standard solution in test tubes in duplicate. To each tube add 5.0 ml of Basal Medium Stock Solution and water to make 10 ml. At the same time prepare four tubes containing 5.0 ml of Basal Medium Stock Solution and water to make 10 ml. Place 1.0 ml, 1.5 ml, 2.0 ml, 3.0 ml and 4.0 ml concentration of the sample solution in test tubes in duplicate. To each tube add 5.0 ml of Basal Medium Stock Solution and water to make 10 ml. Place one complete set of standard and sample tubes together in one tube rack and duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121° for 5 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid colour formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating

rate. Aseptically add 0.5 ml of inoculum to each tube so prepared, except two of the four containing no standard solution of Cyanocobalamin (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40° held constant to within ± 0.5° for 16 to 24 hours.

Terminate growth by heating to a temperature not lower than 80° for 5 minutes. Cool to room temperature. Measure the growth of the organism by determining the transmittance at about 530 nm of each of the solutions in the tubes against the blank (2.4.7). Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100 per cent for the uninoculated blank, read the transmittance of the inoculated blank. Disregard the results of the assay if the difference is greater than 5.0 per cent.

With the transmittance set at 100 per cent for the uninoculated blank, read the transmittance of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Estimation of potency. Prepare a standard concentration response curve by the following procedure. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the ml of standard cyanocobalamin solution per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the sample solution. Read from the standard curve the logarithm of the volume of the standard solution corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in ml, of the sample solution to obtain the difference, x, for each dosage level. Average the values of x for each of three or more dosage levels to obtain $\bar{x} = M'$, the log-relative potency of the sample solution.

Determine the quantity in µg, *cyanocobalamin RS* corresponding to the cyanocobalamin in the portion of material taken for assay by the equation

$$\text{antilog}(M) = \text{antilog}(M' + \log R)$$

in which R is the number of µg of cyanocobalamin that was assumed to be present in each mg (capsule or tablet) of the material taken for assay.

2.2.28. Viral Safety Evaluation of Biotechnology Derived Products from Cell Lines of Human or Animal Origin

Viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect) needs to be evaluated and ensured. This chapter covers products derived from *in vitro* cell culture, such as interferons, monoclonal antibodies and recombinant deoxyribonucleic acid (rDNA) derived products including recombinant subunit vaccines. The safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture media constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this chapter is to describe a general framework for virus testing, evaluation of viral clearance procedures and viral contamination prevention measures. Manufacturers should correlate the information presented here to their specific product and its production process.

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: 1) the use of contaminated biological reagents such as animal serum components, monoclonal antibody affinity column; 2) the use of a virus for the induction of expression of specific genes encoding a desired protein; 3) the use of a contaminated excipient during formulation; 4) contamination during cell and media handling; 5) use of contaminated equipment, during the production process. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

Risk Assessment

Since viral safety is a complex issue, it is important to perform risk assessment to understand the risks associated with viral contamination. The principle of the risk assessment is to consider various factors that may influence the potential level of infectious particles in the medicinal product and factors related to the use of the medicinal product that determine or influence the viral risk to the recipients. All testing suffers from the inherent limitation of quantitative virus assays, i.e.,

the inability to detect low viral concentrations with statistical confidence given the limited size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification process is capable of removing and/or inactivating the viruses.

The risk assessment takes into consideration relevant factors, for example:

- the species of origin;
- the organ, tissue, fluid of origin;
- the potential contaminants in view of the origin and history of the raw material including epidemiological data;
- the potential contaminants from the manufacturing process (for example, from risk materials used during manufacture);
- the infectivity and pathogenicity of the potential contaminants for the intended recipients of the medicinal product, taking account of the route of administration of the medicinal product;
- the amount of material used to produce a dose of medicinal product;
- controls carried out on the raw material, during production and on the final product;
- the manufacturing process of the product and its capacity to remove and/or inactivate viruses.
- selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;
- testing the product at appropriate steps of production for absence of contaminating infectious viruses.

Risk assessment tools such as FMEA (Failure Mode and Effects Analysis) should be considered during virus risk evaluation using standard principles. FMEA tool takes into account three factors the severity, occurrence and detectability of the risk factor in the process. Severity is the potential negative impact of a risk factor on product safety and/or efficacy profile. Occurrence is the frequency or the likelihood that the cause of the failure/contamination will happen. Detectability is likelihood that cause will be detected prior to product release. The ranking system for detectability is the reverse of the severity and occurrence ratings. Risk Priority Number (RPN) is a quantitative method for determining the level of risk by multiplying severity, occurrence and detectability rankings. Rating of 10, 5 and 1 can be considered for assigning the risk of high, medium and low respectively. Severity is assumed high for viral contamination and therefore a constant number of 10 can be assigned for it.

The potential sources of viral contamination that can be considered for risk assessment for the manufacturing of Drug Substance are listed below and in Table 1:

1. Difficult to screen raw materials
2. Susceptible expression/production system
3. Open upstream process
4. Master Cell Bank (MCB) testing
5. Working Cell Bank (WCB) Testing
6. Testing at *in vitro* cell age/EPC
7. Inadequate downstream purification process
8. Testing at unprocessed bulk

I. Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses with appropriate controls to ensure adequate sensitivity and specificity. If presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or non-human primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The list of test to be performed on MCB, WCB and cells at the limit, their detection capability as well as limitation is summarized in Table 2.

Tests for Retroviruses

Tests for viruses including infectivity assays should be performed in sensitive cell culture and electron microscopy (EM) studies for the MCB and for cells cultured up to or beyond the limit of *in vitro* cell age. If infectivity is not detected and no retrovirus or virus like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be non-infectious.

In vitro Assays

In vitro tests are carried out by the inoculation of a test article into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested but should include a human and/or a non-human primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

Table 1– Parameter for risk assessment of viral safety on the manufacturing of drug substance

Parameter	Category	Remarks
Raw material	Animal or human derived raw materials	Animals or human derived raw materials will have high possibility of virus contamination.
	Non animal or human derived raw materials	Animals or human derived raw materials will have low to medium possibility of virus contamination.
	Well documented source	-do-
	Undocumented source	Animals or human derived raw materials will have high possibility of virus contamination.
	Complete viral testing	Animals or human derived raw materials will have low to medium possibility of virus contamination.
	Incomplete viral testing	Animals or human derived raw materials will have high possibility of virus contamination.
Expression/production system	Mammalian cells: Murine, CHO	These cell lines will have high probability of virus contamination.
	Insect cells	-do-
Upstream process	Non bioreactor	Non bioreactor will have high probability of virus contamination as compared to the closed bioreactors.
	Bioreactors	-do-
Downstream process	Does not have orthogonal viral reduction steps	With increased number of orthogonal steps, risk of virus contamination decreases.
	Includes virus filter and one additional virus reduction step	-do-
	Includes virus filters and multiple orthogonal viral reduction steps	-do-
MCB Testing	Virus detected/not detected	Presence of viruses, virus like particles or retrovirus like particles will have higher possibility of viral contamination. Incomplete testing may pose risk of viral contamination.
	Complete/incomplete testing	-do-
WCB Testing	Virus detected/not detected	Presence of viruses, virus like particles or retrovirus like particles will have higher possibility of viral contamination. Incomplete testing may pose risk of viral contamination.
	Complete/incomplete testing	-do-
In vitro cell age/EPC testing	Virus detected/not detected	Presence of viruses, virus like particles or retrovirus like particles will have higher possibility of viral contamination. Incomplete testing may pose risk of viral contamination.
	Complete/incomplete testing	-do-
Unpurified bulk testing	Virus detected/not detected	Presence of viruses, virus like particles or retrovirus like particles will have higher possibility of viral contamination. Incomplete testing may pose risk of viral contamination.
	Complete/incomplete testing	-do-

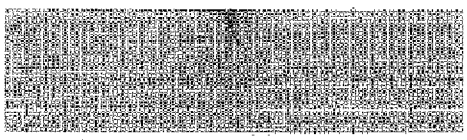


Table 2 – Virus tests, their detection capability and limitations

Test	Test Article	Detection Capability	Detection Limitation	Stage of Testing				
				MCB[1]	WCB	Cells at the limit[2]	EPC	Unprocessed bulk
Antibody production (Examples: RAP, MAP, HAP)	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system	+[7]	-	-	-	-
In vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system	+	-[6]	+	+	-
In vitro virus screen for: 1. Cell bank characterization 2. Production screen	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article) 2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system	+	-[6]	+	+	+
Transmission Electron Microscopy (TEM)	1. Cell substrate: Viable cells 2. Cell culture supernatant	Virus and virus-like particles	Qualitative assay with assessment of identity	+[3]	-	+[3]	+[3]	+
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples	+[4]	-	+[4]	+[4]	+
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system	+	-	+	+	-
Co-cultivation								
1. Infectivity endpoint	Viable cells	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system	+	-	+	+	-
2. TEM end point	Viable cells	Infectious retroviruses	Qualitative assay with assessment of identity	+	-	+	+	-
3. Reverse transcriptase endpoint	Viable cells	Infectious retroviruses	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples	+	-	+	+	+
Polymerase chain reaction (PCR)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious	+	-	+	+	+
	Other virus specific test [5, 8]							

[1] *See text—Master cell bank.*

[2] *Cells at the limit: Cells at the limit of in vitro cell age used for production.*

[3] *May also detect other agents.*

[4]Not necessary if positive by retrovirus infectivity test.

[5]As appropriate for cell lines which are known to have been infected.

[6]For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCBs subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on the cells at the limit of in vitro cell age.

[7]e.g., MAP, RAP, HAP-usually applicable for rodent cell lines.

[8]e.g., tests for cell lines derived from human, non-human primate, or other cell lines as appropriate.

In vivo Assays

A test article should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

Antibody Production Tests

Speciesspecific viruses present in rodent cell lines may be detected by inoculating test article into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. Absence of antibody is generally expected from these tests.

Virus Testing

It is important that all the raw materials used for cell line, MCB and WCB generation as well as in the production process shall be of animal free components and does not pose any risk of viral contamination. If any raw materials used are of animal origin the appropriate testing shall be ensured for such raw materials from manufacturers and shall come with appropriate certifications.

A. Cell Line Qualification

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus. It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. The acceptability of cell lines containing viruses other than endogenous retroviruses can be considered based upon a risk/benefit analysis. This may include nature of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the drug substance.

(a) Master Cell Bank

Viruses can be introduced into the MCB by several routes such as: 1) derivation of cell lines from infected animals; 2) use of virus to establish the cell line; 3) use of contaminated biological reagents such as animal serum components; 4) contamination during cell handling.

Extensive screening for both endogenous and non-endogenous viral contamination should be performed on the MCB. Testing for non-endogenous viruses should include *in vitro* and *in vivo* inoculation tests and any other specific tests, including species specific tests such as the MAP test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

(b) Working Cell Bank

Each WCB should be tested for adventitious viruses either by direct testing or by analysis of cells at the limit of *in vitro* cell age, initiated from the WCB. Antibody production tests are usually not necessary for the WCB. If non-endogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of *in vitro* cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

(c) Cells at the Limit of In Vitro Cell Age/End of Production

The limit of *in vitro* cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial scale conditions to the proposed *in vitro* cell age or beyond using the MCB or the WCB. Cells at the limit of *in vitro* cell age/EPC should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB to provide assurance that the production process is not prone to contamination by adventitious viruses. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

Unprocessed Bulk

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the un-processed bulk would constitute of fluids harvested from the bioreactor. A representative sample of the unprocessed bulk, removed from the production bioreactor prior to further

processing, represents one of the most suitable step at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing.

It is advisable that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. *In vitro* screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious viruses have been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions should be taken.

B. Manufacturing Process Validation

For cell substrates of human or animal origin, virus removal or inactivation processes, individually and overall, should be shown to be able to remove/inactivate any contaminating viruses and to ensure viral safety in the drug substance.

Where appropriate, validation studies should be undertaken using small-scale studies with carefully selected model viruses in order to evaluate the virus clearance/inactivation capability of selected process steps and of the overall process, aiming at a significant safety margins. The results will indicate the extent to which these contaminants can theoretically be inactivated and removed during purification.

Process validation should include the collection and evaluation of data throughout production in order to establish scientific evidence that a process is capable of consistently delivering a quality drug substance. It generally includes collection of data on an appropriate number of production batches. The number of batches can depend on several factors that include, but are not limited to:

i) the complexity of the process being validated;

ii) the level of process variability; and

iii) the amount of experimental data and/or process knowledge available on the specific process.

Process conditions (e.g. column loading capacity, column regeneration and sanitization, height) should be appropriately evaluated. Columns should also be evaluated throughout their expected lifespan with regard to their purification ability (e.g. impurity clearance, collection of intended variants), leaching of ligands (e.g. dye, affinity ligand) and/or chromatographic material (e.g. resin). Process validation activities should normally include the evaluation of resin lifetime, including maximum cycles and/or maximum time duration, using small-scale studies to ensure proper performance and integrity of the columns. In addition, the results should normally be verified at full scale through the life-cycle of the product. These studies should also confirm the suitability of the column cleaning, storage and regeneration procedures.

Where hold times are applied to intermediates (e.g. harvest, column eluate), the impact of hold times and hold conditions on the product quality (e.g. degradation) should be appropriately evaluated.

Evaluation of selected steps (e.g. steps for which high impurity or viral clearance are claimed) operating in worst-case and/or challenging conditions (e.g. maximum hold times, spiking challenge) could be performed to demonstrate the robustness of the process. Depending on the relevance of the experimental model with regard to the final process (e.g. scale, materials, equipment, operating conditions), these studies could be leveraged in support of process validation and/or quality control data requirements.

The information provided in the dossier in support of process validation usually contains both commercial-scale process validation studies and small scale studies. Process validation batches should be representative of the commercial process, taking into account the batch definition as detailed in the process description.

Process changes at the level of fermentation and/or purification during progression to full-scale commercial production may have considerable consequences for the quality of the product, the yield and/or quantitative and qualitative differences in impurities. Consequently the contribution of data from small-scale studies to the overall validation package will depend on demonstration that the small-scale model is an appropriate representation of the proposed commercial scale. Data demonstrating that the model is scalable and representative of the proposed commercial process should be provided. Successful demonstration of the suitability of the small-scale model can enable manufacturers to propose process validation with reduced dependence on testing of commercial-scale batches. Scientific rationale or reference to guidelines can be an appropriate justification to conduct certain studies (e.g. viral removal) only at small scale.

In order to demonstrate viral safety of purification processes used to manufacture drug substance for clinical trials, in-house data from previous validation studies may be used. If in-house experience with highly robust and well understood process steps is available, it may be justified to reduce the product-specific validation effort.

2. Evaluation and Characterization of Viral Clearance Procedures

Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk.

Assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

Provided below are guidelines for process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. In exceptional rare cases where unknown/pathogenic viral contamination is observed then process can be allowed if it has validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Following notes shall be considered for process evaluation of viral clearance:

1. Results for the virus test for cell substrate and/or at the unprocessed bulk level shall be evaluated. Cell cultures used for the production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.
2. The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable if benefits from the product outweigh the risk associated with the product.
3. Virus has been observed by either direct or indirect methods and is believed to be nonpathogenic.
4. When a virus cannot be classified by currently available methodologies, is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic unless its requirement is justified.
5. The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified drug substance.
6. The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved.
7. The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed.

A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general.

"Relevant" Viruses and "Model" Viruses

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall

into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown *in vitro* to sufficiently high titer), a specific "model" virus should be used as a substitute. Appropriate specific "model" virus may be a virus closely related to the known or suspected virus, having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in Appendix 1 and Table 3.

Table 3 – Examples of viruses which have been used in viral clearance studies

Virus	Family	Genus	Natural Host	Genome	Envelope	Size (nm)	Shape	Resistance[1]
Vesicular stomatitis virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 x 150	Bullet	Low
Parainfluenza virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100-200	Pleo/Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80-110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60-70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50-70	Pleo/Spher	Low
Pseudo-rabies Virus	Herpes		Swine	DNA	yes	120-200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25-30	Icosahedral	Med
Encephalomyo-carditis Virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	no	25-30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	DNA	no	60-80	Spherical	Med
SV40	Paoova	Polvomavirus	Monkey	DNA	no	40-50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine Murine	DNA	no	18-24	Icosahedral	Very high

[1]Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory. Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

(a) Facility and Staff

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work (BSL-II and III) and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

(b) Scale-down Production System

The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results. The validity of the scaling down should be demonstrated.

(c) Analysis of Step-wise Elimination of Virus

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to inprocess material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (Appendix 2).

(d) Physical Removal versus Inactivation

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

(e) Inactivation Assessment

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2." The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

(f) Function and Regeneration of Columns

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate or the stability of the viral

clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

(g) Specific Precautions

1. Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.
2. Consideration should be given to the minimum quantity of virus which can be reliably assayed.
3. The study should include parallel control assays to assess the loss of infectivity of the virus due to reasons such as dilution, concentration, filtration or storage of samples before titration.
4. The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test protein sample is no longer identical to the product obtained at commercial scale.
5. Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.
6. Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.
7. Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has antiviral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have antiviral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.
8. Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.
9. Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies: Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in cases (b) to (e) under the heading "Design and Implications of Viral Clearance Evaluation and Characterization Studies", it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained e.g. using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See Appendix 2 for calculation of virus reduction factors and Appendix 3 for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

(i) The appropriateness of the test viruses used;
(ii) The design of the clearance studies;
(iii) The log reduction achieved;
(iv) The time dependence of inactivation;
(v) The potential effects of variation in process parameters on virus inactivation/removal;
(vi) The limits of assay sensitivities;
(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of viruses which

influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 log or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final prod-uct is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.
2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.
3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 log), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.
4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 log10 infectious units per milliliter by a factor of 8 log10 leaves zero log10 per ml or one infectious unit per ml, taking into consideration the limit of detection of the assay.
5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.
6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see Appendix 4).

F. Re-evaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

3. Measures to Prevent Viral Contamination

Physical viral barriers may be employed directly at the points of raw material use (i.e. cell culture medium). High temperature short time (HTST) method inactivates the viruses and certain other microbes by treating the cell culture media with high heat (~102°) for a short period of time (typically 10 seconds). This can act as an in process point of use viral barrier for cell culture processes.

Gamma irradiation has been demonstrated as a viable method of viral inactivation, particularly mitigating against the adventitious virus in animal serum. However this method does not mitigate the risk of adventitious agent exposure that may occur throughout the supply chain as it cannot be employed as point-of-use barrier.

UV-C light (100-280 nm) method for viral inactivation in upstream cell culture process can be employed. Application of 245 nm light to cell culture medium can be effective in damaging viral nucleic acids and proteins however it is typically less effective against enveloped viruses.

4. Summary

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

a) Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

b) Assessment of risk by determination of the human tropism of the contaminants;

c) Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

d) Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

e) Performance of studies that assess virus inactivation and removal.

Appendix 1

Examples of Useful "Model" Viruses

1) Nonspecific "model" viruses representing a range of physico-chemical structures:
   - SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, non-enveloped viruses;
   - a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
   - a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

   These viruses are examples only and their use is not mandatory.

2) For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

Examples of Viruses That Have Been Used in Viral Clearance Studies

Several viruses that have been used in viral clearance studies are listed in Table 3. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers can consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

Appendix 2

Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the log10 of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step or the process. If the following abbreviations are used:

Starting material : vol v′; titer 10a′;
Virus load : (v′) (10a′),
Final material : vol v″ titer 10a″;
Virus load : (v″) (10a″),

The individual reduction factors Ri are calculated according to

$$10Ri = (v/s)\,(10a')\,/\,(v'')\,(10a'')$$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titra-tions, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

Appendix 3

Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

1) Assumptions

   Measured or estimated concentration of virus in cell culture harvest = 10^6/ml

   Calculated viral clearance factor = $>10^{15}$

   Volume of culture harvest needed to make a dose of product = 1 L (10^3 ml)

2) Calculation of Estimated Particles/Dose

$$= \frac{(10^6 \text{ virus units/ml}) \times (10^3 \text{ ml/dose})}{\text{clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{clearnace factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

Appendix 4

Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1) Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2) Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3) The 95 per cent confidence limits for results of within assay variation normally should be order of ± 0.5 log 10 of the mean. Within assay variation can be assessed by standard textbook methods. Between assay variation can be monitored by the inclusion of a reference preparation the estimate of whose potency should be within approximately 0.5 log10 of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4) The 95 per cent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses if the 95 per cent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 per cent confidence limits for the reduction factor are

$$\pm\sqrt{S^2 + a^2}$$

Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per litre) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$$P = \left(\frac{V - v}{V}\right)^n$$

Where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and "n" is the absolute number of infectious particles statistically distributed in V.

If V » v, this equation can be approximated by the Poisson distribution:

$$P = e^{-cv}$$

Where, c is the concentration of infectious particles per litre.

or,
$$c = \frac{\ln p}{-v}$$

As an example, if a sample volume of 1 ml is tested, the probabilities *p* at virus concentrations ranging from 10 to 1,000 infectious particles per litre is:

c	10	10	1000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per litre, in 37 per cent of sampling, 1 ml will not contain a virus particle. If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 per cent are desirable. However, in some instances, this may not be practical due to material limitations.

2.2.29. Microbiological Examination of *Burkholderia cepacia* Complex in Nonsterile Products

NOTE — The following chapter is for information and is not an official requirement

Introduction

The tests described here will allow determination of the absence of *Burkholderia cepacia* complex (Bcc), that may be detected under the conditions described.

These tests are carried out to find out whether a substance or preparation complies with an established specification for microbiological quality and/or to evaluate whether products - especially those for inhalation use or aqueous preparations for oral, oromucosal or nasal use – contain members of the Bcc.

Following procedures are carried out as described under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9).

i) The preparation of sample.

ii) Inactivation of antimicrobial property, if present, in the product.

iii) Confirmation that the surface active substances if used are not toxic for the test-organisms and are compatible with the inactivating agent.

Growth promotive, Inhibitory and Indicative properties of the media and validity of the test

Test each batch of medium prepared either from the dehydrated medium or from the ingredients described for growth promotion. Use the already tested and approved medium prepared from dehydrated medium or from the ingredients as a positive control. Verify the properties of relevant media as described in Table 1.

Preparation of the inoculum

Microorganisms used. *Burkholderia cepacia* ATCC 25416 or NCTC 10743, *Burkholderia cenocepacia* ATCC BAA-245, *Burkholderia multivorans* ATCC BAA-247 or NCTC 13007, *Staphylococus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027.

In order to prevent any phenotypic changes in the strains used, the organisms used in the test should not be more than 5 passages made from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium.

Grow each of the aerobic bacterial strains separately in Casein soyabean digest broth or on Casein soyabean digest agar and incubate them at 30° to 35° for 18 to 24 hours. After incubation, re-suspend the growth of each of the organisms separately in *buffered sodium chloride-peptone solution pH 7.0*. Use suspension of these organisms within 2 to 4 hours, or within 24 hours if stored at 2° to 8°. Use inoculum not more than 100 CFU for growth promotion and validity of test method.

Negative control. To test the sterility of the medium and the diluent, use the medium, *Burkholderia cepacia* selective Agar (without organisms) as a negative control. There should not be any growth of microorganisms in this control. If a negative control fails (microorganisms grow in the control), its cause should be investigated.

Tests for growth promotion by solid media. Using the surface spread method as described under *Total viable aerobic count* in *Microbial contamination in nonsterile products* (2.2.9), inoculate each plate with not more than 100 CFU of appropriate microorganism (Table 1). Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth obtained on the medium should be comparable to that on the same medium previously approved.

Tests for growth inhibition in solid media. Inoculate the appropriate medium with not less than 100 CFU of the appropriate microorganism (Table 1). Incubate at the specified temperature for not less than the longest period of time specified in the test. There should not be any growth of the microorganism on this medium.

Tests for indicative properties. Using the surface spread method as described under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9). Inoculate each plate with not more than 100 CFU of appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colony

Table 1– Growth promoting, inhibitory and indicative properties of media

Test/Medium	Property	Test Strains
Test for *Burkholderia cepacia* complex (Bcc)		
Burkholderia cepacia selective Agar	Growth promoting + Indicative	*Burkholderia cepacia, Burkholderia cenocepacia or Burkholderia multivorans*
	Inhibitory	*Pseudomonas aeruginosa, Staphylococcus aureus*

morphology and indication reactions should be similar to that obtained with the previously approved batch of medium.

Validity of the test method. The ability of the test to detect Bcc in the presence of the product to be tested must be established. The incubation time for the method suitability should not exceed the shortest incubation period specified. Suitability must be confirmed if there is a change in testing performance or a change in the product that may affect the outcome of the test.

For each new product to be tested prepare a sample as described below in the pertinent paragraph under Testing of Products. At the time of mixing add each test organism in the prescribed growth medium. Inoculate not more than 100 CFU of the test organisms individually. Carry out the test as described under Testing of Products, using the shortest incubation period prescribed. The Bcc microorganism must be detected with the colony morphology and indication reaction as described.

If the growth in presence of product is inhibited by a factor more than 2, it indicates that product has antimicrobial activity, then it should be inactivated as mentioned in *inactivation of antimicrobial activity under appropriateness of enumeration method in presence of product in Microbial contamination in nonsterile products* (2.2.9).

Testing of Products

Sample Preparation and Pre-Incubation

Using Casein soyabean digest broth (Medium 1) as a diluent, make 1 in 10 dilution of not less than 1 g of the product to be examined as mentioned under *Total aerobic viable count* in *Microbial contamination in nonsterile products* (2.2.9) and use 10 ml or the quantity corresponding to 1 g or 1 ml of the product to inoculate a suitable amount (determined as under *Validity of the test method*) of Casein soyabean digest broth, mix and incubate at 30° to 35° for 48 to 72 hours.

Sub-culture on a plate of *Burkholderia cepacia* selective Agar (BCSA) and incubate at 30° to 35° for 48 to 72 hours. A greenish-brown colonies with yellow halos, or white colonies surrounded by a pink–red zone on BCSA indicates the possibility of presence of *Burkholderia cepacia* Complex. This should be confirmed by identification tests.

If there is no growth of such type of colonies, or identification tests are negative it indicates absence of *Burkholderia cepacia* Complex and the product passes the test.

Recommended solutions and culture media

The following solutions and culture media have been found to be satisfactory for the purpose for which they are prescribed in the Pharmacopoeia. Other media may be used provided their suitability can be demonstrated.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 ml

Sterilise in an autoclave.

Casein soyabean digest broth

Pancreatic digest of casein	17.0 g
Papaic digest of soyabean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Dextrose monohydrate	2.5 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2.

***Burkholderia cepacia* Selective Agar**

Casein peptone	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Sodium chloride	5.0 g
Yeast extract	1.5 g
Phenol red	0.08 g
Gentamicin	10.0 mg
Vancomycin	2.5 mg
Crystal violet	2.0 mg
Polymyxin B	600,000 U
Agar	14.0 g
Purified water	1000 ml

Add the ingredients without the antibiotics. Adjust the pH so that after sterilisation it is 6.8 ± 0.3 at 25° temperature. Sterilize in an autoclave, cool the medium to 45° to 50°. Before use add a 1 per cent solution of the sterile filtered antibiotics, mix, and pour into the plates.

2.2.30. Approach to Alternative Microbiological Methods

NOTE — The following chapter is for information and is not an official requirement

Introduction

This chapter provides guidance on the evaluation, and use of alternative microbiological methods in place of compendial methods for evaluation of the quality of pharmaceutical products.

The microbiological methods described in Indian Pharmacopoeia include; effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in nonsterile products (2.2.9); microbiological assay of antibiotics (2.2.10) and sterility test (2.2.11). These microbiological tests methods are based on the growth of organisms and hence are time consuming. For instance, current Sterility test procedure takes 14 days to get the result. The results from these methods cannot be used to take any corrective action in time.

Alternative microbiological methods have shown potential for real-time or near real-time results with the possibility of earlier corrective action. These new methods, if validated and adapted for routine use, can also offer significant improvements in the quality of testing.

Alternative methods may be used for raw materials, in-process samples, finished pharmaceutical products, for environment monitoring and for utilities such as water, steam and compressed gasses or wherever applicable for testing of microorganisms etc.

In this chapter, alternative microbiological methods for pharmaceutical products are described. For available methods, the basic principle, the advantages and disadvantages of the method are discussed.

It is not the purpose of this chapter to recommend one method/technique over another, nor is it the intention to provide an exclusive or exhaustive list of alternative methods that can be used for pharmaceutical microbiological control.

The information provided herein may be used, in the process of choosing an alternative microbiological method as a supplement or as an alternative to pharmacopoeial microbiological methods and to give guidance on validation of the chosen method.

Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. Automated procedures utilizing the same basic principles as the test procedures given in the monograph may also be used to determine compliance. Such alternative or automated procedures must be validated and are subject to approval by the authority competent to authorised manufacturer of substance or product.

In the event of doubt or dispute, the methods of the Pharmacopoeia are alone authoritative and only the result obtained by the procedure given in the pharmacopoeia is conclusive.

1.1. Current Pharmacopoeial Methods

There are three types of determinations in Microbiology methods as described in Pharmacopoeia.

1. Qualitative test for the presence/absence of microorganisms.
2. Quantitative test where the microorganisms are enumerated on growth medium.
3. Identification methods where the specified organisms are identified based on genotypic, phenotypic and biochemical tests.

1.2. Alternative Microbiological Methods

Alternative microbiological methods employ direct and indirect methods of detection. Amplification of the signal or peak is achieved by the instruments.

Alternative methods for the control of microbiological quality are divided into categories as following:

1.2.1. Growth based methods

1.2.1.1. General critical aspects of methods based on early detection of growth

This method depends on microbial growth to provide an indication of the presence and/or number of microorganisms. Typically low levels of microbial contamination of the products detection may take 24 h or longer. Increased sensitivity can be achieved with filtered products. In this case, after filtration, the membrane filter is incubated on the medium and the result is expressed as quantify in the quantity corresponding to the filtered volume. These systems, if they use an incubation step in liquid media, do not offer quantitative information, but a presence/absence determination in the quantity analysed. Analysis of more than one sample quantity may offer a semi-quantitative estimation (limit test). The major benefit of early detection methods compared to classical methods is often the capacity to simultaneously process a large number of samples and the potential to obtain a result in a shorter time.

The methods described below (1.2.1.2 to 1.2.1.5) can be used for quantitative, semi-quantitative and qualitative analysis. These methods are non-destructive and subsequent identification of microorganisms is also possible.

1.2.1.2. Electrochemical methods

Microorganisms multiplying and metabolising in appropriate growth media produce highly charged ionic metabolites from weakly charged organic nutrients leading to the modification of electrical properties in such media. These changes in impedance (measured by conductance or capacitance) are monitored with electrodes included in the culture vessels and in contact with the culture medium. The measurable end-point is the time taken to detect a predetermined impedance change. For particular types of microorganisms, the detection time is inversely proportional to the initial inoculum size. For yeasts and moulds, which only produce small changes in electrical

impedance, an indirect measurement of conductance can be used. Direct measurement of capacitance can also be carried out.

There is no direct relationship between the original microbial level and the detectable end-point. This method may be used in microbiological assay of antibiotics, effectiveness of antimicrobial preservatives, and presence/absence of testing.

1.2.1.3. Measurement of consumption or Production of Gas

These methods detect microbial growth either by changes in the electrical properties of a sensor in response to a change in gas composition or by colorimetric changes of a sensor in response to physicochemical changes in the growth medium in contact with that sensor. The systems are based on non-destructive techniques which enable subsequent identification or strain typing of the microorganisms. Bacteria and/or fungi may be grown in closed containers and continuous monitoring can be performed using automated instruments that measure gas evolution (e.g.CO_2) or consumption (e.g. O_2) as surrogate markers of microbial growth. Furthermore, the production of metabolites or elimination of nutrients can lead to changes in pH or redox potential. All of these changes can be measured either directly or indirectly as changes in colorimetric or suitable markers in the growth medium.

There is no direct relationship between the original microbial level and the detectable end-point.

The incubation temperature, the physiological state and type of microorganism, the initial load and the algorithm for data processing can significantly affect the results or the time to detection.

These methods can be used for presence/absence testing of filterable or non-filterable samples.

1.2.1.4. Bioluminescence

Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP first needs to be released from the microorganisms using an appropriate extractant, followed by an assay using the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The signal-to-noise ratio can be increased by addition of ADP and converting this ADP into released ATP.

Qualitative Test: Microorganisms are cultivated in liquid medium. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) (e.g. bioluminescence in a tube or a well of a microtitre plate). The RLU obtained from the sample is compared with a pre-determined threshold value. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value.

Quantitative Test: Microorganisms are captured on a membrane and cultivated by incubation on agar medium. Using a charge coupled device (CCD) camera, the ATP released from microcolonies can be detected by light emission and a quantitative determination is possible.

If the sample has a high level of bacterial contamination, the detection is rapid. For low levels of contamination, it is necessary to increase the number of microorganisms using an incubation step in culture media (liquid or solid). The yield of ATP varies from one microorganism to another and can depend on several factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age. Additional factors such as turbidity, sample colour or product matrix effects can also influence bioluminescence measurements. Extraction of ATP is generally a destructive process which should be considered with respect to any subsequent need for identification of detected microorganisms.

This method can be used for presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill), total aerobic microbial count (TAMC), environmental and water monitoring, testing for effectiveness of antimicrobial preservatives.

1.2.1.5. Turbidimetry

Microbial growth leads to detectable changes in medium opacity, which can be accurately quantified by optical density measurements at a specified wavelength. In its simplest form, such measurements are performed using a standard spectrophotometer, generally over a wavelength range of 420-615nm. Alternative automated systems employ microtitre plate readers offering a continuous readout with early detection of optical density change.

Attempts have been made to extrapolate the initial microbial contamination from the time to detection, but this is limited to healthy microorganisms with reproducible growth characteristics.

This method can be used for determination of inoculum size of microbial suspension, microbiological assay of antibiotics and testing for effectiveness of antimicrobial preservatives.

1.2.2. Direct measurement

1.2.2.1. Solid Phase Cytometry

Microorganisms are stained for viability by exposure to a conjugated, initially non-fluorogenic, fluorophore. An intact cellular membrane is required to retain and accumulate the fluorophore within the cytoplasm. Inside metabolically-active microbial cells, the conjugate is enzymatically cleaved and the fluorescent derivative is released intracellularly. Microorganisms are collected on a membrane filter either before or after viability staining.

Membrane surfaces retaining vital-stained cells are then scanned by a laser beam and epifluorescent excitation allows

the detection of single, viable fluorescent microorganisms. Appropriate software allows differentiation of viable microorganisms from auto fluorescent particles. The high sensitivity and rapidity of the method permit detection of microbial contaminants within a few hours. Total cell counts (viable and non-viable) can be obtained using fluorescent staining. Metabolically active, fastidious and viable non-culturable microorganisms can all be detected. This may result in reappraisal of the microbial limits established for the samples under evaluation. Spores require initiation of germination to enable detection. Single cell detection may be achievable, but identification of isolates might not be possible. False positives may occur due to autofluorescent particles that can be difficult to differentiate from microorganisms. Signal discrimination and enhancement can be aided by microcolony growth.

This rapid and sensitive method is use for the non-specific evaluation of microbial contamination.

1.2.2.2. Flow cytometry

Fluorophore-labelled microorganisms can be detected in suspension as they pass through a flow cytometer. Viable microorganisms can be differentiated from non-viable particles by use of a viability-indicating fluorophore (see *1.2.2.1*). The cell suspension stream is dispersed into a narrow channel and exposed to a laser which excites the fluorophore. Micro-organisms and particles are then counted in different channels depending on whether or not they contain a fluorescent cell.

Direct flow cytometry may be applied to the microbiological analysis of both filterable and nonfilterable materials, and after possible enrichment in the case of the low contamination levels. It gives near real-time detection, but is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it is often necessary to add an incubation step in culture media, in which case the method becomes a combination of a growth-based method and a direct detection method. Particle size and number may have a significant effect on performance, in this condition samples may require serial dilution. With the exception of filterability, similar considerations to those in solid phase cytometry apply. Clumping of microorganisms can be a problem.

In contrast to solid phase cytometry, this method offers the potential to detect and enumerate microbial contamination in materials containing particulate matter and if the material cannot be filtered. If a pre-incubation step is needed, the method becomes a qualitative determination.

1.2.2.3. Epifluorescent filter technique

This technique may be considered a forerunner of solid phase cytometry. Microorganisms, concentrated by filtration of the sample, are stained with a fluorescent dye (formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI)), that can be detected by epifluorescent illumination. Fluorescent vital staining techniques, fluorescent redox dyes such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be used to highlight respiring cells. Coupled with microscopy, the method allows rapid detection of microorganisms with an absolute sensitivity that is dependent on the volume of product filtered and the number of fields of view examined. Semi-automated auto-focusing systems coupled to image analysis have served to improve the utility of this method. A modification of the principle involves sampling using an adhesive sheet (which permits collection of cells from surfaces), subsequent staining on the sheet itself, followed by direct observation using an epifluorescence microscope.

The distribution of microorganisms on the membrane affects method robustness. The intensity of fluorescence can be influenced by the staining process and the metabolic status of the microorganisms. Fluorescence is not necessarily an indicator of viability. A brief period of culture on the filter surface prior to staining allows microcolony formation; these microcolonies stain readily, can be easily enumerated and are demonstrable evidence of viability.

Direct Epifluorescent Filter Technique (DEFT) is generally limited to low viscosity fluids, although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Monitoring of microbial contamination has been successfully applied to aqueous pharmaceuticals.

1.2.3. Cell component analysis

1.2.3.1. *Phenotypic Techniques*

1.2.3.1.1. Immunological Methods

Antibody-antigen reactions can be employed to detect unique cellular determinants of specific microorganisms. These reactions can be linked to agglutination phenomena and colorimetric/chromogenic or fluorimetric and other suitable end-points, which offer both quantitative and qualitative detection. Enzyme-linked immuno sorbent assays (ELISA) offer simple solid-phase methodologies.

Immunological detection methods depend on the unique expression of specific identifiers, but do not necessarily demonstrate the presence of viable microorganisms.

This method used for the detection and identification of specified microorganisms.

1.2.3.1.2. Fatty Acid Profile

The fatty acid composition of microorganisms is stable, well conserved and shows a high degree of homogeneity within different taxonomic groups. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted, the occurrence and amount of the resulting fatty acid methyl esters are measured using high-

resolution gas chromatography. The fatty acid composition of an unknown isolate is compared with a database of known isolates for a possible match and identification.

The use of fatty acid profiles for microbial identification requires a high degree of standardisation. It is critical for the fatty acid composition of microbial cells that isolates are grown using standardised media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must also be employed, with frequent runs of calibration standards and known isolates being very important.

This method used for identification or characterization of environmental and product microbial contamination (for contaminant tracing and detection of specified microorganisms).

1.2.3.1.3. Fourier transforms infrared (FTIR) spectroscopy

A Fourier transformation of the infrared spectrum of whole microorganisms gives a stable, recognisable pattern typical of the taxonomic groups of microorganisms. The analysis of the FTIR pattern can be performed with commercially available instruments. The isolate is grown on a standard medium and harvested. Cell mass is transferred to a carrier, and the infrared spectrum is recorded. The Fourier transformation is calculated and the pattern is compared with a database of known isolates for a possible match and identification.

The use of FTIR patterns for microbial identification requires a high degree of standardisation. It is critical for the FTIR pattern of microbial cells that isolates are grown using standardised media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed, and particular attention must be paid to this in the validation process.

This method used for Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified microorganisms).

1.2.3.1.4. Mass spectrometry

Ionised particles released by exposing microbial isolates to a laser in a vacuum can be analysed by mass spectrometry, providing characteristic spectra. Similarly, intact microbial cells, when subject to intense ionisation under matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, release a distinctive pattern of charged species. Such spectra can be compared with known profiles.

The isolates must be cultured under standardised conditions prior to analysis.

This method is used for identification or characterisation of environmental and product microbial contaminants (for contaminant tracing and detection of specified microorganisms).

1.2.3.2. Genotypic Techniques

Identification and detection of microorganisms as well as characterisation of strains belonging to the same species may be achieved by direct detection of nucleotide target sequences that are unique for a particular microbial species or microbial group, and are targets of the genotypic (DNA or RNA-based) detection techniques. These detection techniques may be separated into 3 broad categories: direct hybridisation, nucleic acid amplification and genetic fingerprinting.

1.2.3.2.1. Direct hybridization

DNA probes are short, labelled, single-strand segments of DNA that hybridise with a complementary region of microbial DNA or RNA. The probe or target DNA is usually labelled with either radioactive, fluorescent or chromogenic molecules in order to provide a hybridisation signal. Hybridisation assays include fluorescence in-situ hybridisation (FISH) and microarray-based techniques.

Hybridisation generally requires a large amount of the target DNA for analysis, which may result in lower detection sensitivity. The availability of suitable probes may be limited.

Due to the high specificity of the sequence-based hybridisation reaction, this method may be used for both detection and identification of microorganisms.

1.2.3.2.2. Nucleic acid amplification technique

This technique relies on the reiteration of the DNA polymerisation process, leading to an exponential increase of a specific nucleic acid fragment. The polymerase chain reaction (PCR) is the most widely used method for target DNA amplification. In this cyclic process, a specific DNA fragment is copied by a thermo stable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers, previously designed to flank the target sequence and to hybridise with it (2.8.1).

After PCR, the amplified nucleic acid targets can be analysed using several methods of post- amplification analysis: fragment size analysis in gel electrophoresis, DNA sequencing or specific detection by hybridisation with a fluorescent-labelled probe. Real-time PCR eliminates the need for further post-amplification processing and offers the additional advantage that the likelihood of cross-contamination is minimised.

An important advantage of real-time PCR is the ability to quantify the starting amount of the DNA target sequence in the original sample, in contrast to conventional PCR techniques, which are based on end-point detection.

Due to the high sensitivity and specificity of amplification techniques, they are suitable for both detection and identification of microorganisms. Real-time PCR is needed for quantitative or semi-quantitative analysis of the target. Besides

quantitative determinations, the real-time PCR technique allows simultaneous detection of multiple targets in a single sample, as long as appropriate primers and probes that allow for multiplexing are employed. The sequencing of different genes (e.g. 16S rDNA, 23S rDNA, rpoB, Gyr) is best applied to the identification of microorganisms.

1.2.3.2.3. Genetic fingerprinting

Genetic fingerprinting is the identification of a strain on the basis of its DNA profile (or RNA for RNA viruses). Individual DNA profiles may be different due to genetic diversity between strains of the same species, and the aim of the fingerprinting methods is to discriminate between these strains. The classical genetic fingerprinting technique characterizes microorganisms using restriction fragments of chromosomal DNA from bacterial and fungal genomes.

Different strains from the same species may exhibit different patterns and these differences are referred to as restriction fragment length polymorphisms (RFLPs). As cutting the chromosomal DNA with restriction enzymes generates too many fragment bands to be efficiently and accurately compared, several modifications of the conventional RFLP-based method have been developed. Examples of the kind of technologies used are ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Several other fingerprinting methods use PCR to selectively amplify defined subsets of DNA restriction fragments from the entire genome, for example random amplified polymorphic DNA (RAPD) and variable number tandem repeats (VNTR).

All fingerprinting techniques require that the microorganism is present as a pure culture. Depending on the method, a preliminary enrichment cultivation step may be necessary if a defined quantity or a specific DNA preparation is required for the test.

Genetic fingerprinting methods are mainly used for strain discrimination (characterization below species level). They are a powerful tool for investigating and tracing the source and the spread of microbial contamination.

2. Validation of Alternative Microbiological Methods

2.1. Validation Process

Two levels of validation must be carried out for the application of alternative microbiological methods, namely primary validation and validation for the intended use. The manufacturer of the alternative technology typically performs primary validation of a method, whereas validation for the intended use, which is a verification of the suitability or applicability of the method in a given situation, must be seen as the responsibility of the user.

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified.

2.1.1. Description of the technique

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the manufacturer. Through primary validation, the method must be fully detailed with respect to the conditions required for application, the materials and equipment needed and the expected signal. The user shall critically review the available information.

2.1.2. Risk-Benefit Analysis

For validation of specific alternative microbiological methods, it is critical that the purpose of the quality assurance procedure is precisely outlined, as this defines the type and depth of information needed. The information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis. The risk level in adopting an alternative method varies depending on the technology considered, the methodology it replaces, the nature of the measurements taken (qualitative, quantitative or identification), the particular product or process attribute being evaluated, the location of the measurement in the manufacturing process chain and various other factors.

Risk analysis tools may be utilised in order to determine which alternative method is to be implemented, to assist in the justification of its implementation or to better understand the impact of implementation on production and/or product quality. An alternative method can be justified for use if the information obtained gives a scientifically sound measure of microbiological quality, and if the limitations of the method are not more severe than those of the pharmacopoeial method.

2.1.3. Primary validation

The manufacturer, using a panel of test microorganisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

- Prerequisite treatment of sample or microorganisms;
- Type of response;
- Specificity;
- Detection limit;
- Quantitation limit;
- Range;
- Linearity;
- Accuracy and precision;
- Robustness of the method in a model system.

Verification of primary validation data given by manufacturer

The method is verified using the panel of test microorganisms given by the corresponding pharmacopoeial chapter. The alternative method must be applied according to the specified procedure of the manufacturer, without the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in the model system used by the manufacturer.

2.1.4. Validation for intended use

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

- User requirement specification (URS);
- Design qualification (DQ);
- Installation qualification (IQ);
- Operational qualification (OQ);
- Performance qualification (PQ).

2.1.4.1. User Requirement

Organizations wishing to develop and validate alternative method produce a user requirement specification document. This document should include all critical functions of the technology, critical user interface requirements, space requirements, environmental requirements, operational requirements, and all other important characteristics of an alternative method for the intended use. These requirements will be specific to the company or organization, as well as to the alternative method's intended use, and therefore the requirements should be generated by user.

Instrument Qualification. Most alternative microbiological methods will depend on specific equipment. This analytical equipment is subject to industry standard instrument qualification requirements.

Validation of alternate technologies. The basic rationale for using an alternative methodology is to improve on some aspect of the existing technology of the current compendial method without sacrificing essential characteristics of that technology (e.g., plate count and membrane filtration). The current technology for compendial microbiology methods consists of detection of the growth of viable microorganisms on (or in) a nutrient medium. The alternative technology must be at least equivalent to the current technology in terms of performance for the intended use.

Method suitability. This consideration must address both the technology's suitability to the specific test and the lack of product inhibition and enhancement on the test results.

Suitability of the technology to the specific test. Many compendial microbiological tests have mandated test requirements. The test results are frequently used to determine compliance with finished product specifications, and the specifications are dependent on sample volume or quantity, the alternative technology must be able to satisfy sample volume requirements as required in the general test method. The use of a lesser volume or sample size is not recommended and would need to be fully justified by the user on a case-by-case basis. The alternative technology is considered suitable if it can meet all critical parameters of the compendial test.

Inhibition and enhancement. Specific products may interfere or enhance the signal of different measurement technologies to the specific signal of interest. This component of alternative microbiological method validation (i.e., suitability) must be demonstrated for each product.

Preparation of the User Requirement Specification document. Input from all stakeholders for the alternate microbiological test method is required. These stakeholders may include representatives from the Microbiology, Quality, Regulatory Affairs, and Operations groups, as well as others. The time spent on this step should be considered an investment in reaching a clear understanding of the company's needs before equipment is purchased, which will drive the performance qualification. At minimum, this document should include the following:

- Purpose and intended use (defined need for instrument);
- Description of who will use the equipment;
- Operational requirements (data format, user interfaces, and operating environment);
- Constraints (timetables, downtime, maintenance, user skill levels, product compatibility, limit of detection, accuracy, and rapidity);
- Life cycle (development, testing, delivery, validation, training, and obsolesce);
- Capability (turnaround time, test capacity and throughput, and labor requirements);
- Sustainability (consumables, calibration, validation, and preventative maintenance).

The user may need to tailor the specific recommendations in to their particular instrument qualification specifications.

2.1.4.2. Design qualification (DQ)

The DQ provides documented evidence that the design of any associated equipment is suitable for correct performance of the method. Most alternative method systems are based on commercial off-the-shelf equipment. The DQ is most suitably performed, therefore, by the instrument developer/manufacturer. Nevertheless, the user shall verify that the equipment meets the specifications laid down in the URS for the intended application.

2.1.4.3. Installation qualification (IQ)

The IQ provides documented evidence that the equipment has been provided and installed in accordance with its specifications.

2.1.4.4. Operational qualification (OQ)

The OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures.

2.1.4.5. Performance qualification (PQ)

The PQ provides documented evidence that the method, with the equipment installed and operated according to operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method. This is typically done with a panel of microorganisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing microorganisms). This assures that the conditions employed by the user laboratory make it possible to satisfy the criteria described by the manufacturer of the method in the model system used for the primary validation.

Verification for the intended use

The following points, where applicable, should be addressed:

- Compatibility of the response with the sample preparation that the user normally performs for product testing (method suitability testing);
- Limit and range of detection of the method with regard to sample size and sample availability;
- Specificity of the response with regard to the influence of the product ingredients;
- Linearity of the response with regard to the types of samples to be analysed;
- Accuracy and precision of the response with regard to the types of samples to be analysed.

Acceptance criteria for the method will need to be defined as a function of the application and the validation data. Since Alternative Microbiological Method may produce sensitive, accurate results, however while using alternative method, acceptance criteria will change in comparison to conventional methods (Quantitative/Qualitative tests); if this is the case, the responsibility lies with the manufacturer/user to produce/show the similarity/equivalence of the results/acceptance criteria while using alternative methods. Since the Acceptance criteria mentioned for Microbiological quantitative/qualitative tests in IP is conclusive.

2.2. Validation Criteria

The validation parameters generally recommended for qualitative, quantitative and identification microbiological tests. These criteria are described below and summarised in Table 1.

Table 1– Validation criteria for qualitative, quantitative and identification tests

Criterion	Qualitative test	Quantitative test	Identification test
Accuracy	–	+	+
Precision	–	+	–
Specificity	+	+	+
Detection limit	+	+/–	–
Quantitation limit	–	+	–
Linearity	–	+	–
Range	–	+	–
Robustness	+	+	+
Repeatability	+	+	–
Ruggedness	+	+	–
Equivalency	+	+	–

2.2.1. Accuracy

The accuracy of an alternative method is the closeness of the test results obtained by the alternative test method to the value obtained by the compendial method, to be demonstrated across the dynamic (operational) range of the method.

2.2.2. Specificity

The specificity of an alternate qualitative microbiological method is defined as its ability to detect a range of challenge microorganisms specific to the technology. "Range of microorganisms" may be defined as a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are representative in terms of morphological and physiological attributes appropriate for the method and the product.

2.2.3. Limit of Detection

The limit of detection (LOD) of an alternate microbiological method is defined as lowest number of microorganisms in a defined volume of sample that can be detected, but not necessarily quantified, under the stated experimental conditions. This should be conducted with the quality control organisms mentioned in general test methods, such as effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in nonsterile products (2.2.9) and sterility test (2.2.11).

2.2.4. Robustness

A capacity of the method to remain unaffected by small but deliberate variations in method parameters, e.g., reagent volume, incubation time, or ambient temperature providing an indication of its reliability during normal usage. A measure of robustness is not a comparison between the compendial and alternate methods; rather, it is a necessary component of validation of the alternate method so that the user understands the limits of the operating parameters of the method. The user may rely on data supplied by test method manufacturer.

2.2.5. Limit of Quantification

The limit of quantification (LOQ) of an alternate microbiological method is defined as the lowest number of microorganisms in a test sample that can be enumerated with acceptable accuracy and precision under defined experimental conditions.

2.2.6. Ruggedness

The degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions such as different analysts (for example, three), instruments, and reagent lots (the method for demonstration may follow instrument or materials manufacturer recommendations, or it could be based solely on data supplied by test method manufacturer).

2.2.7. Linearity

The ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range.

2.2.8. Range

The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with specified accuracy, precision, and linearity.

2.2.9. Repeatability Precision

The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of the same suspension of microorganisms and uses different suspensions across the range of the test. Also known as "repeatability".

2.2.10. Method suitability

For each new product to be tested using the validated alternate microbiological method, perform the suitability test as described in general test methods, such as effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in nonsterile products (2.2.9); microbiological assay of antibiotics (2.2.10) and sterility test (2.2.11) using the number of unit and quantities prescribed and the sample preparation appropriate for the product and the required test sensitivity to determine the absence of a product effect that would obscure the signal of the method. Method suitability may be demonstrated using three independent tests. Only the accuracy and precision validation parameters are required for quantitative methods. For qualitative methods, recovery of challenge organisms as indicated in microbial contamination in nonsterile products (2.2.9) and sterility test (2.2.11) is sufficient.

2.2.11. Equivalency

All microbiological tests are performed to enable informed decision making regarding the microbiological quality of a product, raw material, component, or process step. In this respect, the intended purpose of microbiological tests may be to either evaluate for the presence or absence of microorganisms (as in the sterility test) or to estimate the number of organisms present. The technological means by which microbiological test methods assess microbiological quality and enable a product quality decision may differ from the growth based means typical of reference methods. The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a Colony Forming Units (CFU), but rather a different approach to obtaining a cell count estimate. Therefore, the validation of alternative microbial methods should involve two components: (1) equivalence demonstration and (2) analytical method and equipment qualification.

2.2.31. Design and Development of Biological Assay and its Validation

Introduction

Purpose and Scope

The purpose of the chapter Development of Biological Assays and its Validation presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and its validation that are fit for their specific use. This general chapter is intended to guide the development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter's recommended methods may be resource intensive during assay development, early implementation can yield benefits.

This chapter is intended for both the practicing bioassay analyst who is engaged in biological assay Development, Validation and Analysis of bioassay, later on the assay uses for lot release of drug Substance, drug product, In-process

sample and its stability testing. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. It is generally useful to assess similarity using the dose response curve, range of linear point on the curve, maximum response etc. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology and variability of assay that can prove challenging to balance with a help of statistical tools.

Biological Assay/ Potency Assay

Biological assays (also called bioassays) are an integral part of the quality assessment required for the manufacturing and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g. animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests. Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product.

Since the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency/ activity is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically similar, statistical similarity (a consequence of the Test and Standard similarity) should be present and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unit less measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100 per cent). The Standard can be a material established as such by a national (e.g., IPC) or international (e.g. WHO) organization, or it could be an internal Standard.

Development of Biological Assay

Use of Bioassay

In-process and process Intermediate

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. The bioassay's precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product's known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay's estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity. For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification.

Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

Basics of Biological assay

***In-Vitro* (Cell-Based) Bioassays**

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines or engineered cell lines transfected with appropriate receptors. Additionally, non-transformed cell lines which can be maintained over a sufficient number of passages (e.g. fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages to be set. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of receptor and signalling mechanisms and longer stability. The cellular responses to the protein of interest

depend on the drug's MOA (Mechanism Of Action) and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion and enzyme activation. Assays involving these responses may require incubation of the cells over several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signalling mechanism–such as second messengers assay, protein kinase activation, or reporter gene expression–have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample's specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, EC_{50} of the concentration–response curve or the response range (maximum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e. it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g. multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay's cell line should be documented, including details of the cell line's history from origin to banking. For example, for a recombinant cell line this might include the identification of the source of the parental cell line (internal cell bank, external repository, etc.) of the DNA sequences used for transfection; and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include

a. Identity (e.g., isoenzyme, phenotypic markers, genetic analysis);
b. Morphology (e.g., archived photographic images);
c. Purity (e.g., mycoplasma, bacteria, fungus and virus testing);
d. Cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions);
e. Thaw viability (immediately after being frozen and after time in storage);
f. Growth characteristics (e.g., cell doubling times); and
g. Functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment.

The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays:

a. Cell type (adherent or non-adherent);
b. Cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells);
c. Culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation);
d. Incubation conditions (temperature, CO_2 , humidity, culture times from thaw);
e. Cell harvesting reagents and techniques (for adherent cells, method of dissociation);
f. Cell sorting & cell counting;
g. Determination of cell health (growth rate, viability, yield);

h. Cell passage number and passaging schedule;

i. Cell line stability (genetic, receptor, marker, gene expression level); and

j. Starvation or stimulation steps.

This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

***In-Vivo* Bioassays**

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration–response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoeitin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples. Some assay methods lend themselves to the use of colony versus naïve animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. For the test for pyrogenicity a validated Monocyte Activation test (MAT) (refer 2.2.25) may also be used in lieu of the rabbit pyrogen test. If pyrogen test is used for testing highly antigenic compounds for pyrogenicity, native animals should be used to avoid generating inaccurate or confounded results.'

'If animals recently introduced to the colony fail to respond as expected after several administration of a compound, they should be segregated from the colony so that they do not cause future invalid or indeterminate assay results.

Ethical euthanasia provision or rehabilitation of the animals thus segregated shall be considered as per CPCSEA guidelines.'

'Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine. Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal's state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitization, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

***Ex-Vivo* Bioassays**

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells. Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of *in vivo* assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

Reagent qualification

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard's biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the

bioassay may be used to qualify a cell culture reagent such as fetal bovine serum and other sterile non-animal culture media. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

Reference Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100 per cent) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release. A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., -70° or -20°, instead of 2° – 8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilizable formulation or the addition of carrier proteins such as human serum albumin, stabilizers, etc.). The Standard material should be tested for stability at appropriate intervals.

System suitability criteria of the bioassay such as maximum or background response, EC_{50} slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability. At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay's assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the Primary Standard. Subsequent Standards can be prepared using current manufacturing processes and can be designated Working Standards. Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.

Statistical Aspects of Bioassays

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assays those results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

Assumptions for data similarity

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed similarity for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard–Test sample pair that passes the assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration–response curves. It may be reasonable to use a biological assay system to describe or even compare

response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptably low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Assay variance, weighting and transformation

For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; variance heterogeneity may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are the estimate of relative potency may still be reasonable; however, failure to address non-constant variance around the fitted concentration–response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate and standard errors and confidence intervals for all parameters (including a Fieller's Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Variance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay. Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen for an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically.

Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale. Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, logit of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the Power of the Mean (POM) function. A POM coefficient of $k = 2$ corresponds to a log transformation of the data.

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of responses observed in a specific assay. In practice, many bioassays have relatively large variation in log EC_{50} (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log EC_{50} induces what appears to be large variation in response near the mean log EC_{50}, often yielding too-low weights for observations near the EC_{50}.

If the assay is fairly stable (low variability in EC_{50}), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be proportional to the function of concentration where this plot

approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modelling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the EC_{50} parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the EC_{50}. A weighted model with low weights for concentrations near the EC_{50} would misrepresent a major feature of such an assay system.

Linearity of Concentration–Response

The bioassay data responses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to determine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity in such case the significant level should be evaluated (95 per cent to 99.99 per cent) during development of the assay and shall be validated during validation of bioassay. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency. If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity.

Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result.

The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve. The problem is more complex in assays where there is even modest variation in the shape or location of the concentration– response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section Fixed and Random Effects in Models of Bioassay Response).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation. How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the

parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed. For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoids. Such functions require a sufficient number of concentrations or dilutions to fit the model.

To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote. A linear model is sometimes selected because of apparent efficiency and ease of processing. *Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile.* For data that follow a four-parameter logistic model, these are the concentrations near the centre of the response region, often from 20 per cent to 80 per cent response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Assay Suitability Testing

System Suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or perhaps a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g. residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99 per cent or more i.e. 99.99 per cent for linearity) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or simulation studies that measure the influence of changes in a parameter on potency estimation. Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

System Suitability

System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of potency. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters. Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on sensitivity analysis (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable.

The System Suitability should check at least for parallelism, linearity and regression for linear/ nonlinear models. The significant level for validity test should be evaluated during development and refined during the validation.

— Parallelism at 95 per cent;
— Linearity (lack of fit) at 99.99 per cent;
— Regression at 95 per cent.

Sample Suitability

Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an EC_{50} (and potency) within the range of the assay system.

Similarity

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and non-symmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

Range

The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to determine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of EC_{50}s from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estimated relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference EC_{50}.

Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias. An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum's excursive value is due to human error or instrument malfunction, then the datum should be omitted from calculations.

In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. *Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.* Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used; the bioassay may be expected to be the most prone to outlying data. The management of outliers is appropriate with bioassay data on at least two levels: where an individual datum or a group of data (e.g., data at a concentration, including blank) can be checked against expected responses for the sample and concentration; and, separately, when estimates of relative potency from an assay can be checked for consistency with other independent estimates of the potency of the same material. Three important aspects of outlier management are prevention, labelling, and identification. Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur.

In effect, the error never becomes an outlier because it is prevented from occurring. Good practice calls for the examination of data for outliers and labelling ("flagging") of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlying datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory's investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier's cause is not a clear indication that statistical outlier testing is warranted.

Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests. Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected. Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

Replication based approaches

When replicates are performed at concentrations of a Test sample and the Standard, an "extra variability" (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM (Power of Mean) approach. Where heterogeneity exists non-normality is likely present, and the range rather than standard deviation or RSD should be used. The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair (n = 2) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

Model based approaches

Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make fewer assumptions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful. Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data's centre exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers.

Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels or all levels of interest are discretely present like sample, concentration, temperature and duration of thaw and incubation time. Data for a response at some level or combination of levels of a fixed factor can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor. Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day but there is interest in predicting the variation in response associated with the factor "day". Examples of random effects include reagent lot, operator or day if there is no interest in specific reagent lots, operators or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated. Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled to properly include those factors in the design and then to

include other factors as random. If the factor may switch from random to fixed effect or vice versa. The factor should normally be modelled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect. Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that include multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

Bioassay Development Process

For the assays requiring biological system, due consideration shall be given to non-animal test methods. In case an animal test is preferred over a non-animal test method given in the monograph, using the animal model shall be justified.

Whenever animal tests are used these shall be established in such a way that animal usage is minimized to the extent possible.

In the present text, development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

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An objective of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the concluded development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine analysis. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent, technician qualification and calibration of working reference standard.

Identification of Critical reagent/ equipment

It is necessary to understand the mechanism of action of drug for the intended bioassay. Based on the MOA of drug/ product, select the reliable source during development stage for like:

i. Cell line as cell substrate for In-vitro assay/ animal species for In-vivo model assay;
ii. Foetal bovine serum;
iii. Growth media/ supplementary enzymes etc.;
vi. Detection dye/ Kit etc.;
v. Type of plates;
vi Equipment etc.

After addressing the initial required materials during development, the lot of the material should be qualified and implemented in routine analysis.

Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g. good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns or from the edge to the centre of the plate and are often called plate effects. Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a uniformity trial in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration–response curve, is used in all wells of the plate. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g. of sample by concentration combinations) in adjacent wells should be avoided.

Blocking is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped

by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A complete block design occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An incomplete block design occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Randomization is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

A layout that provides some protection from plate effects and can be performed manually is a strip-plot design, samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left). Assay variance may then be addressed, as necessary, by increased assay replication (increased number of plates in an assay).

A split-plot design, an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row. Such a strategy may be difficult to implement even with the use of robotics.

Dilution Strategy

Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors. It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability.

Design of Experiments

Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that affect bioassay performance. Assay development using DOE may proceed through a series of steps: response and risk analysis; screening; response optimization; and confirmation.

Response and risk analysis

Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.

Screening and response optimization

Once potential key factors have been identified from response and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional

factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions about design selection and analysis to ensure accurate identification of experimental factors.

A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

Confirmation

The Assay Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

Data Analysis During Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria.

The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don't need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

Step 1. Choose an appropriate statistical model. Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis.

Step 2. Fit the chosen statistical model to the data without the assumption of parallelism and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance. Transform the data as necessary or, if needed, choose a weighting scheme.

Step 3. Screen for outliers, and remove as is appropriate. This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. It is necessary to screen the raw data for outliers before attempting to fit the model. During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation; outlier test(s) applied, and results there from. Note that outlier procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

Step 4. Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.

Step 5. If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear.

Step 6. Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

Bioassay Validation

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance

characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. IP general chapter 2.5.10 "Validation of Analytical procedures" and ICH Q2 (R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products; their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice. Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. This chapter provides validation goals pertaining to relative potency bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include relative accuracy, specificity, intermediate precision, and range. Laboratories may use dilutional linearity to verify the relative accuracy and range of the method. Although robustness is not a requirement for validation, this chapter recommends that a bioassay's robustness be assessed prior to validation. In addition, describes approaches for validation design (sample selection and replication strategy), validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed with reference to pre-validation experiments performed to optimize bioassay performance

Concept of Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a "suitable for intended use" validation to support release of clinical trial material, and a final, comprehensive validation prior to any regulatory filing. Preliminary system and sample suitability controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise.

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use.

Specificity. Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.

Relative accuracy and Range. In order to establish the relative accuracy and range of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency).

Intermediate Precision (IP). The potential influences on the bioassay from inter-run factors such as multiple analysts, instruments or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the intermediate precision (IP) of the bioassay. An appropriate study of the variability of the potency values obtained, including the impact of intra-assay and inter-run factors can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the reportable value (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay.

Robustness. Robustness studies are usually performed during bioassay development, key factors in these studies such as incubation time and temperature and for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g. a temperature sensitive reagent that varies in its sensitivity from lot-to-lot).

Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy the intended validation parameters and justified target acceptance criteria for each parameter and a proposed data-analysis plan. In addition, assay, run and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and can be updated with data from the validation.

Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in

order to support changes to the method. The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g. variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to pre-validation experiments may be included as part of the validation study report. Pre-validation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from pre-validation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For relative accuracy, sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product.

A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the range of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target analyte, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data. The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run. The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a reportable value for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent runs of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments {including those that implement formalized design of experiments (DOE)} with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability.

During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format. A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters' results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to satisfy the assumptions of the statistical methods.

Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are relative accuracy, specificity, IP (which incorporates repeatability), and range. Other parameters discussed in general chapter (2.5.10. Validation of Analytical Procedures) and ICH Q2 (R1) such as detection limit and quantitation limit has not been included because they are usually not relevant to a bioassay that reports relative potency. Linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

Specificity

For products matrices and different but related biopharmaceutical molecules, specificity involves demonstrating no significant interference from matrix components or different but related biopharmaceutical molecules. This can be assessed via parallel dilution of the Standard and matrix or different but related biopharmaceutical molecules, if there is no significant response for matrix or different but related biopharmaceutical molecules and the response confirms to the no interference from matrix or different but related biopharmaceutical molecules.

For identical assay, response can be assessed via parallel dilution of the Standard and identical sample if the curves are similar and the potency conforms to expectations of a Standard-to-Sample comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests.

Relative Accuracy (Dilutional Linearity)

The relative accuracy of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a dilutional linearity study. The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in relative bias across levels. The relative bias at individual levels is calculated as follows:

$$\text{Relative bais} = \left(\frac{\text{Measured Potency}}{\text{Target Potency}} - 1 \right) \text{per cent}$$

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in relative bias across levels, the estimated relative bias at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol.

Intermediate Precision

Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the Intermediate Precision (IP) of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots. Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a variance component analysis on the validation results. Variance component analysis is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML). A variance component analysis yields variance component estimates such as corresponding to intra-run and inter-run variation.

$$\text{Per cent CV (intra assay)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

and

$$\text{Per cent CV (inter assay)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

These can be used to estimate the IP of the bioassay, as well as the variability of the reportable value for different bioassay formats (format variability). IP expressed as percent coefficient of variation (per cent CV).

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

Range

The range of the bioassay is defined as the true or known potencies for which it has been demonstrated that the

analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypo-potent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

Bioassay Maintenance and Assay Transfer

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining statistical process control (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability.

Analysis of Bioassay

Most of the potency determination assays are relied on biological assay where the chemical characterization assay does not mimic the exact mechanism of action of the drug for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. In biological assays, the variable is the amount of drug, the system is a cell, and a response is a property of the cell that changes when the drug is given. The experimental design employed in biological assays is different from those typical used in chemical and physical experiments since the intrinsic variation in living matter increases the complexity of the experiment and the data analysis. The experimental design has not only to take into account the intrinsic variability in the biological test units but also other factors as operators, day variation, batch variation, etc.

Principle of Biological assay

A biological assay or bioassay is used to determine the effect of a substance/product on a certain type of living matter. A biological test system, for example animals, tissue, etc, is exposed to a particular stimulus like a drug, whose concentration (dose) is usually varied. The magnitude of the response of the biological system depends on the dose. In contrast with physical or chemical methods, detailed information of the drug activity as a function of the dose is obtained. A special characteristic of bioassays is that one of the largest sources of variation in the outcome is the difference between the test units, and since the response is dependent on living matter this introduces large variability between measurements obtained by identical operations. The response can be a characteristic like a count, number, body weight of the test units, or a change in a characteristic, change in body weight, or the occurrence of a certain phenomena, e.g. viability or death.

Biological assays are usually comparative. The capacity of a substance to cause a specific effect is estimated relative to a standard. The standard and the test preparations are identical in their biological activity principle and differ only in extends to which they are diluted by solvents. This type of bioassay is used in the pharmaceutical industry to determine the potency of test product relative to a standard sample.

Introductory step for analysis of Biological assay

Ensure the method development & validation activity should be completed and the preliminary information of the assay is fixed to avoid further decision and changes and also it will help in investigation of any nonconformity.

Analysis of biological assay should be analyse base on protocol where

- Cell line handling and passage range are the most critical step for biological assay. Select the validated range of passage for analysis, where the responses are reproducible for shape, EC_{50}; range of upper asymptote and lower asymptote.
- Based on assay model, for linear model select a linear region (Choosing model) of the curve for the standard and sample (subset of data) to be used in the determination of the relative potency using the predefined scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
- Screen the data for detection of potential outliers, as chosen during development or as per defined procedure, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.
- Determine which potential outliers are to be removed and fit the model to be used for System suitability assessment. The approach for the outlier removal should be fixed during development and shall be part of the procedure for maximum valid outlier in assay curve etc. If a large number of outliers are found above that expected from the defined procedure, that calls into question the assay. After removal of outlier, the assay does meet the system suitability; invalid the assay and repeat the assay based on justification for the occurrence.
- System suitability: System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. The significant level for linearity must evaluated as most precise result may fails at lower significant level (P= 0.05) i.e. 95 per cent, increase the significant level to (P= 0.0001) 99.99 per cent may serve the problem. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, IC_{50} (or EC_{50}); and variation around the fitted model.
- Sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as "Fails Sample Suitability.". Relative potencies for other test samples in the assay may still be reported. Most prominent of sample suitability criteria is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than EC_{50} (or IC_{50}).
- Once the Assay system suitability and sample suitability meet the (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.
- If the assay are more variable and often a single assay is not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay procedure or monograph, before determining a final estimate of potency and a confidence interval.

For replacement of missing data, most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph or method analysts generally do not need to replace missing values.

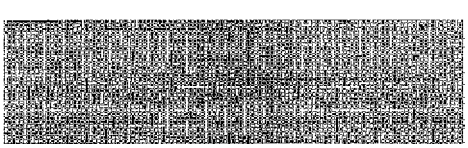
Statistical Analysis Models

Many statistical tools computer based/ statistical software can be successfully used to describe a concentration–dose response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered. Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models.

This section presumes that decisions were made to fix statistical model during development, checked during validation, and then use for routine analysis.

Improving or Updating Bioassay System

A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving



or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved.

2.2.32. Subvisible Particulate Matter in Therapeutic Protein Injections

This chapter can be used as an alternative to 2.5.9 general chapter "Particulate contamination". It specifically addresses therapeutic protein injections and related preparations, allowing use of smaller test product volumes and smaller test aliquots to determine particulate matter content, with sample-handling instructions that take into account the issues associated with the analysis of these materials. Although the methodology and limits presented in this chapter are preferred for therapeutic protein injections, the use of regulatory-acceptable alternative analytical methods with adequately developed subvisible particulate limits is acceptable.

Therapeutic protein injections are biotechnology-derived products of protein or peptides as defined in Genrel requirement for Biotechnology derived therapeutic products, volume III and other therapeutic protein injections such as naturally sourced therapeutic proteins, including their final infusion preparations. Certain preparations that are not amenable to these tests, such as prophylactic vaccines, are not included in this guidance.

Particulate matter in therapeutic protein injections consists of mobile undissolved substances that may originate from various sources. The particles may be (a) truly foreign, or "extrinsic", e.g., unexpected foreign material, such as cellulose; (b) "intrinsic" resulting from addition or by insufficient cleaning during manufacturing, such as tank metals or gaskets, lubricants, filling hardware, or resulting from instability, e.g., changes over time, such as insoluble drug salt forms or package degradation; and (c) "inherent", such as particles of the protein or formulation components. These entire particle types may be detected and counted in the test method described in this chapter. In addition, for light obscuration (LO) methods, gas bubbles are commonly counted during particulate matter testing and are counting artifacts. For the determination of subvisible particles in therapeutic protein injections, the Light Obscuration Particle Count Test is the preferred method. The Microscopic Particle Count Test similar to that described in 2.5.9 may be useful in cases where the particles being analyzed are primarily nonproteinaceous, or in cases where examination of particle characteristics may be important. The results from this test are not equivalent to those from the LO test.

Not all therapeutic protein injections can be examined directly for subvisible particles by the LO method. When the test method is not directly applicable to specific test samples, as indicated by issues with method verification (reproducibility, linearity), a quantitative dilution with an appropriate diluent can be made to allow analysis by light obscuration. Method verification may be required to ensure the appropriateness of the sample handling procedures and the performance of the method for each drug product.

In the tests described below, the results obtained by examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, sampling plans based on known operational factors must be developed to support valid inferences from observed data in order to characterize the level of particulate matter in a large group of units. Sampling plans should be based on considerations of product volume, particle numbers historically found in comparison to limits, particle size distribution, and variability of particle counts between units. Products that are used with a final filter during administration (in-line) are exempt from these requirements, providing that scientific data are available to justify the exemption.

Microscopic Particle Count Test

As noted, the LO method is the preferred method for therapeutic protein injections and parenteral infusions. However, the microscopic method may be used when appropriate, such as determination of extrinsic and intrinsic particle types only. It should be demonstrated, however, that particular classes of particles (e.g., inherent) are also being counted when using this method. For the determination of product acceptability, apply the limits for the membrane microscopic test in general chapter 2.5.9 Because of the interference of some protein particles and their physical characteristics (fragile or translucent), the results of the Microscopic Particle Count Test are not equivalent to those of the Light Obscuration Particle Count Test, and the two methods cannot be considered interchangeable.)

The preparation meets the requirements of the test if it contains particles within the maximum limits shown in Table 1.

Table 1

Limits:

Sample	Particle size in µm (equal to or greater than)	Maximum number of particles
Preparations in containers with nominal content of more than 100 ml	10	Average in the units tested 12 per ml
	25	2 per ml
Preparations in containers with nominal content of 100 ml	10	3000/container
	25	300/container
Less than 100 ml	10	3000 per container
	25	300 per container
Ophthalmic injections	10	50 per ml
	25	5 per ml
	50	2 per ml

Light Obscuration Particle Count Test

A suitable instrument that is based on the principle of light blockage and that allows an automatic determination of particle size and number.

The sensor selected must be appropriate for the intended particle size range and anticipated particle count. Several standardization steps such as sample volume accuracy, sample flow rate, sensor resolution, calibration, and particle count accuracy are important for the proper operation of the apparatus and are further reviewed in general information chapter 2.5.9. The instrument should be calibrated with suitable certified reference materials consisting of dispersions or spherical particles of sizein the range between 2 and 100 µm. The materials should be dispersed in particle-free water, taken care to prevnt aggregation of particles.

General Precautions

Carry out the test under conditions that limit ingress of particulate matter, preferably in a laminar-flow cabinet. The glassware and equipment used should be properly cleaned and handled to remain particle free. Care should be taken to minimize shaking and other stresses to the preparations (for liquids or reconstituted powders) and to prevent introduction of air bubbles into the preparation under examination. This is particularly important when preparations are combined or transferred to the container in which the determination will be carried out.

Preparatory Steps

Glassware selected for the method should accommodate the volume of blank and sample aliquots, allow easy mixing, and be amenable to repeated cleaning used to prevent any significant contribution to particle counts. Typically, separate vessels are utilized and retained specifically for this test only. Cleaning and rinsing of the method glassware should be in close proximity to the counting area.

The work area and equipment should be separated from general lab traffic. A good supply of filtered water (to comply with Particle-Free Water) and support equipment should be maintained and available for the routine work. Refer to chapter 2.5.9.

Two types of system controls are recommended: a Blank Test and System Suitability Verification.

1. The Blank Test involves the following:
   A. Run at the start of the day or analysis to ensure that proper care has been used in preparing the site and apparatus for use (Environmental Blank).
   B. Any additional blanks run throughout the day (such as between product families) should also comply with the Blank Test requirements (Procedural Blank).
2. The System Suitability Verification is run to verify proper count/ml and ratio for the current Particle Count RS or equivalent commercial standard. See chapter 2.5.9.

Blank Test

In order to check that (a) the environment is suitable for the test, (b) the glassware is properly cleaned, and (c) the water or suitable solvent that will be used is particle free (b and c constitute the "blank"), the following test is carried out. Determine the count in five aliquots of degassed particle-free water or suitable solvent, representative of the product test volume (but not less than 1 ml). If the number of particles 10 µm or greater in size exceeds 1 particle per ml for the combined aliquot volume, the precautions taken for the test are not sufficient. Repeat the preparatory steps until the environment, glassware, and water are suitable for the test determination. The Blank Test must be run initially each day (environment) and is recommended throughout the daily routine, especially between product families (procedural).

System Suitability Verification

To verify system suitability, *Particle-Free Water* and the Particle Count Reference Standard or equivalent commercial standard aqueous suspension are tested from the same or similar type of container as product samples, are degassed in the same manner, and generally are handled like the test article. Ensure that the system can meet the Blank Test requirement. Determine the particulate matter in a sample of the Particle Count Reference Standard that corresponds to the product test volume, according to the method described below. Results from the Standard preparation should fall within the stated specifications of the Reference Standard.

Method

Product samples are tested in the manner that most suitably represents delivery (e.g., expelled syringe contents). For parenteral that have a sufficient volume (i.e., volume large enough to facilitate testing), testing of individual units is often diagnostic. For parenteral products that do not have a sufficient volume, carefully and thoroughly mix each unit, and then combine the contents of a suitable number of units in a separate container to obtain the volume required for a single test (generally 0.2 to 5.0 ml). If a dilution is performed, ensure that the blank container has sufficient volume for product and diluents and that few, if any, particles are introduced in the process. Open each unit cautiously, remove the sealing closure, and avoid contamination of the contents before sampling, diluting, or, if necessary, pooling.

When a product solvent is specified, e.g., for lyophilized solids or powders for parenteral use, the reconstitution or dilution must be performed with the appropriate amount of specified solvent. In this case, the solvent itself may be tested to ensure that it is not a significant source of particles. Subtraction of the solvent particle count from the total count is not allowed.

Eliminating gas bubbles is a key step, especially for proteinaceous products that readily entrain gas. Two methods are recommended: either allowing the product fluid to stand under ambient pressure or applying a gentle (e.g., 75 Torr) vacuum. Other methods may be used when demonstrated to be suitable. Sonication should be avoided.

Once the samples have been degassed, they must be remixed gently to suspend all particles by mixing the contents of the sample gently but thoroughly by an appropriate means, e.g., slow swirling of the container by hand. Inversion to mix the product fluid is not recommended at any time.

Immediately after mixing, withdraw not less thanfour aliquots, each of a volume appropriate for the instrument's capacity (generally 0.2 to 5.0 ml). Count the number of particles over the selected size range, including particles equal to or greater than 10 and 25 µm. Disregard the result obtained for the first aliquot, and calculate the mean number of particles at each size range for the remaining aliquots of the preparation being tested. A tare volume also can be applied to control sampling that should be representative of the sensor dynamic range and needle volume.

General Considerations

Dilution is allowed as long as the diluent and methods are demonstrated to be appropriate and the smallest level of dilution that allows for reproducible testing is used. Under certain circumstances dilution of samples may be required to obtain reliable results. This may be the case with high-concentration products and/or high-viscosity products that cannot be drawn into the instrument properly; products that cause saturation of the instrument sensor due to high counts, especially in the smaller than 10-µm size ranges; and cases where the high concentration results in very small differences in refractive index between the solution and the protein aggregates, etc. In situations where sample dilution is conducted, the underlying rationale for the dilution as well as the suitability of the selected dilution scheme including choice of diluent must be demonstrated. Suitability of the dilution scheme and of the diluent may be assessed by demonstrating consistency of results over a dilution range. Bridging studies with and without dilution at size ranges intended for particle counting should be considered. Studies exploring the ability of dilution to decrease the number of aggregates, or to increase particles due to changes in the ratio of protein to excipients, should also be performed. An orthogonal technique may be used to justify the suitability of the method by verifying the impact of dilution.

Evaluation

The values below are derived historically from general chapters 2.5.9. If specifications are different from those stated below, they will be indicated in an individual monograph where available.

Products that are used with a final filter during administration (in-line) are exempt from these requirements, providing that scientific data are available to justify the exemption. However, filtrates are expected to comply with the guideline. For products supplied or first reconstituted in <100 ml, and then diluted for infusion in a volume >100 ml, particle content should be assessed both before and after dilution and evaluated based on their final volume.

Table 2

Limits.

Sample	Particle size in µm (equal to or greater than)	Maximum number of particles
Preparations in containers with nominal content of more than 100 ml		Average in the units tested
	10	25 per ml
	25	3 per ml
Preparations in containers with nominal content of 100 ml	10	6000/container
	25	600/container
Less than 100 ml	10	6000 per container
	25	600 per container
Ophthalmic injections	10	50 per ml
	25	5 per ml
	50	2 per ml

The preparation meets the requirements of the test if it contains particles within the maximum limits shown in table 2. If the average number of particles exceeds these limits, test the preparation by Microscopic Particle Count Test.

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Identification

2.3.1. General Identification Reactions of Ions and Functional Groups

The following tests may be used for the identification of chemicals referred to the Pharmacopoeia. They are not intended to be applicable to mixtures of substances unless so specified.

Acetates

A. Heat the substance under examination with an equal quantity of *oxalic acid*; acidic vapours with the characteristic odour of acetic acid are liberated.

B. Dissolve about 30 mg of the substance under examination in 3 ml of *water* or use 3 ml of the prescribed solution, add successively 0.25 ml of *lanthanum nitrate solution*, 0.1 ml of *0.1 M iodine* and 0.05 ml of *dilute ammonia solution*. Heat carefully to boiling, within a few minutes a blue precipitate or a dark blue colour is produced.

Acetyl Groups

In a test-tube (about 180 mm x 18 mm) place 10 to 20 mg or the prescribed quantity of the substance under examination and add 0.15 ml of *phosphoric acid*. Close the tube with a stopper through which passes a small test-tube (about 100 mm x 10 mm) containing *water* to act as a condenser. On the outside of the smaller tube, hang a drop of *lanthanum nitrate solution*.

Except for substances hydrolysable only with difficulty, place the apparatus in a water-bath for 5 minutes and remove the smaller tube. Mix the drop with 0.05 ml of *0.01 M iodine* on a porcelain tile or glass slide and then add one drop of *2M ammonia* at the edge of the mixed drop; after 1 or 2 minutes a blue colour is produced at the junction of the two drops and the colour intensifies and persists for a short time.

For substances hydrolysable only with difficulty, heat the mixture slowly to boiling point over an open flame instead of using a water-bath.

Alkaloids

Dissolve a few mg or the prescribed quantity of the substance under examination in 5 ml of *water*, add *dilute hydrochloric acid* until the solution has an acid reaction and then add 1 ml of *potassium iodobismuthate solution*; an orange or orange-red precipitate is formed immediately

Aluminium Salts

Dissolve about 20 mg of the substance under examination in 2 ml of *water* or use 2 ml of the prescribed solution, add about 0.5 ml of *2 M hydrochloric acid* and about 0.5 ml of *thioacetamide reagent;* no precipitate is produced. Add drop wise *dilute sodium hydroxide;* a gelatinous white precipitate is produced which dissolves on further addition of *dilute sodium hydroxide*. Gradually add *ammonium chloride solution;* the gelatinous white precipitate reappears.

Amines, Primary Aromatic

Acidify the prescribed solution with *2 M hydrochloric acid* or dissolve 0.1 g of the substance under examination in 2 ml of *2M hydrochloric acid* and add 0.2 ml of *sodium nitrite solution*. After 1 or 2 minutes, add the solution to 1ml of *2-naphthol solution;* an intense orange or red colour and, usually, a precipitate of the same colour is produced.

Ammonium salts

To 10 ml of 10 per cent w/v solution of the substance under examination or use the prescribed solution add 0.2 g of *light magnesium oxide*. Pass a current of air through the mixture and direct the gas that is evolved to just beneath the surface of a mixture of 1 ml of *0.1M hydrochloric acid* and 0.05 ml of *methyl red solution*; the colour of the solution changes to yellow. On addition of 1 ml of a freshly prepared 10 per cent w/v solution of *sodium cobaltinitrite*, a yellow precipitate is produced.

Antimony Compounds

Dissolve with gently heating about 10 mg of the substance under examination in a solution of 0.5 g of *sodium potassium tartrate* in 10 ml of *water* and allow to cool. To 2 ml of this solution or to 2 ml of the prescribed solution add *sodium sulphide solution* dropwise; a reddish orange precipitate which dissolves on adding *dilute sodium hydroxide solution* is produced.

Arsenic Compounds

Heat 5 ml of the prescribed solution on a water-bath with an equal volume of *hypophosphorous reagent*; a brown precipitate is formed.

Barbiturates, Non-nitrogen Substituted

Dissolve 5 mg of the substance under examination in 3 ml of *methanol*, add 0.1 ml of a solution containing 10 per cent w/v of *cobaltous nitrate* and 10 per cent w/v of *calcium chloride*, mix and add, with shaking, 0.1 ml of *dilute sodium hydroxide solution*; a violet-blue colour and a precipitate are produced.

Barium Salts

Dissolve 20 mg of the substance under examination in 5 ml of *dilute hydrochloric acid* and add 2 ml of *dilute sulphuric acid*; a white precipitate, insoluble in *nitric acid*, is formed.

Benzoates

A. To 1 ml of a 10 per cent w/v neutral solution of the substance under examination add 0.5 ml of *ferric chloride test solution*; a dull yellow precipitate, soluble in *ether*, is formed.

B. Moisten 0.2 g of the substance under examination with 0.2 to 0.3 ml of *sulphuric acid* and gently warm the bottom of the tube; a white sublimate is deposited on the inner walls of the tube and no charring occurs.

C. Dissolve 0.5 g of the substance under examination in 10 ml of *water* or use 10 ml of the prescribed solution and add 0.5 ml of *hydrochloric acid*; the precipitate obtained, after crystallisation from *water* and drying at a pressure of 2 kPa, melts at about 122° (2.4.21).

Bicarbonates

A. Solutions, when boiled, liberate carbon dioxide.

B. Treat a solution of the substance under examination with a solution of *magnesium sulphate*; no precipitate is formed (distinction from carbonates); boil, a white precipitate is formed.

C. Introduce into a test-tube 0.1 g of the substance under examination suspended in 2 ml of *water* or in 2 ml of the prescribed solution. Add 2 ml of *2 M acetic acid*, close the tube immediately using a stopper fitted with a glass tube bent at two right-angles, heat gently and collect the gas in 5 ml of *barium hydroxide solution*; a white precipitate forms that dissolves on addition of an excess of *dilute hydrochloric acid*.

Bismuth Compounds

A. To 0.5 g of the substance under examination add 10 ml of *2 M hydrochloric acid* or use 10 ml of the prescribed solution. Heat to boiling for 1 minute, cool and filter, if necessary. To 1 ml of the filtrate add 20 ml of *water*; a white or slightly yellow precipitate is formed which on addition of 0.05 to 0.1 ml of *sodium sulphide solution* turns brown.

B. To about 50 mg of the substance under examination add 10 ml of *2 M nitric acid* or use 10 ml of the prescribed solution. Heat to boiling for 1 minute, allow to cool and filter, if necessary. To 5 ml of the filtrate add 2 ml of a 10 per cent w/v solution of *thiourea*; an orange-yellow colour or an orange precipitate is produced. Add 4 ml of a 2.5 per cent w/v solution of *sodium fluoride*, the solution is not decolorised within 30 minutes.

Bromides

A. Dissolve a quantity of the substance under examination equivalent to about 3 mg of bromide ion in 2 ml of *water* or use 2 ml of the prescribed solution. Acidify with *dilute nitric acid* and add 0.4 ml of *silver nitrate solution*. Shake and allow to stand, a curdy, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 ml, of *water*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate obtained in 2 ml of *water* and add 1.5 ml of *10 M ammonia*. The precipitate dissolves with difficulty.

B. Introduce into a small test-tube a quantity of the substance under examination equivalent to about 5 mg of bromide ion or the prescribed quantity. Add 0.25 ml of *water*, about 75 mg of *lead dioxide*, 0.25 ml of *acetic acid* and shake gently. Dry the inside of the upper part of the test-tube with a piece of filter paper and allow to stand for 5 minutes. Prepare a strip of suitable filter paper of appropriate size. Impregnate it by capillary, by dipping the tip in a drop of *fuchsin solution decolourised* and introduce the impregnated part immediately into the tube. Starting from the tip, a violet colour appears within 10 seconds that is clearly distinguishable from the red colour of fuchsin, which may be visible on a small area at the top of the impregnated part of the paper strip.

Calcium Salts

A. Dissolve 20 mg of the substance under examination in 5 ml of *5 M acetic acid* or add 1 ml of *glacial acetic acid* to 5 ml of the prescribed solution. Add 0.5 ml of *potassium ferrocyanide solution*, the solution remains clear. Add about 50 mg of *ammonium chloride*; a white, crystalline precipitate is formed.

B. To 5 ml of a 0.4 per cent w/v solution of the substance under examination add 0.2 ml of a 2 per cent w/v solution of *ammonium oxalate*; a white precipitate is obtained that is only sparingly soluble in *dilute acetic acid* but is soluble in *hydrochloric acid*.

C. Dissolve 20 mg of the substance under examination in the minimum quantity of *dilute hydrochloric acid* and neutralise with *dilute sodium hydroxide solution* or use 5 ml of the prescribed solution. Add 5 ml of *ammonium carbonate solution*; a white precipitate is formed which, after boiling and cooling the mixture, is only sparingly soluble in *ammonium chloride solution*.

Carbonates

A. Suspend 0.1 g of the substance under examination in a test-tube in 2 ml of *water* or use 2 ml of the prescribed solution. Add 2 ml of *2 M acetic acid*, close the tube immediately using a stopper fitted with a glass tube bent at two right-angles, heat gently and collect the gas in 5 ml of *0.1 M barium hydroxide*, a white precipitate is formed that dissolves on addition of an excess of *dilute hydrochloric acid*.

B. Treat a solution of the substance under examination with a solution of *magnesium sulphate*; a white precipitate is formed (distinction from bicarbonates).

Chlorides

A. Dissolve a quantity of the substance under examination equivalent to about 2 mg of chloride ion in 2 ml of *water* or use

2 ml of the prescribed solution. Acidify with *dilute nitric acid*, add 0.5 ml of *silver nitrate solution*, shake and allow to stand; a curdy white precipitate is formed, which is insoluble in *nitric acid* but soluble, after being well washed with *water*, in *dilute ammonia solution*, from which it is reprecipitated by the addition of *dilute nitric acid*.

B. Introduce into a test-tube a quantity of the substance under examination equivalent to about 10 mg of chloride ion or the prescribed quantity, add 0.2 g of *potassium dichromate* and 1 ml of *sulphuric acid*. Place a filter-paper strip moistened with 0.1 ml of *diphenylcarbazide solution* over the mouth of the test-tube; the paper turns violet-red. (Do not bring the moistened paper into contact with the potassium dichromate solution).

Citrates

A. To a neutral solution of the substance under examination add a solution of *calcium chloride*; no precipitate is produced. Boil the solution; a white precipitate soluble in *6 M acetic acid* is produced.

B. Dissolve a quantity of the substance under examination containing about 50 mg of citric acid in 5 ml of *water* or use 5 ml of the prescribed solution. Add 0.5 ml of *sulphuric acid* and 3 ml of *potassium permanganate solution*. Warm until the colour of the permanganate is discharged and add 0.5 ml of a 10 per cent w/v solution of *sodium nitroprusside* in *1 M sulphuric acid* and 4 g of *sulphamic acid*. Make alkaline with *strong ammonia solution*, added dropwise until all the *sulphamic acid* has dissolved. On addition of an excess of *strong ammonia solution*, a violet colour, which turns violet-blue, is produced.

Esters

To about 30 mg of the substance under examination or to the prescribed quantity add 0.5 ml of a 7 per cent w/v solution of *hydroxylamine hydrochloride* in *methanol* and 0.5 ml of a 10 per cent w/v solution of *potassium hydroxide* in *ethanol (95 per cent)*. Heat to boiling, cool, acidify with *2 M hydrochloric acid* and add 0.2 ml of a 1 per cent w/v solution of *ferric chloride*; a bluish-red or red colour is produced.

Ferric and Ferrous Salts

Dissolve a quantity of the substance under examination containing about 10 mg of ferrous and ferric compound or to 3 ml of the prescribed solution add *ammonium sulphide solution;* a black precipitate is formed. The precipitate dissolve in cold, *3 M hydrochloric acid* and evolves *hydrogen sulphide*.

Ferric Salts

A. Dissolve a quantity of the substance under examination containing about 10 mg of iron in 1 ml of *water* or use 1 ml of the prescribed solution. Add 1 ml of *potassium ferrocyanide solution;* an intense blue precipitate, insoluble in *dilute hydrochloric acid,* is produced.

B. To 2 ml of solution containing about 0.1 mg of Iron or to 3 ml of the prescribed solution add *1 M sodium hydroxide;* a reddish brown precipitate is formed.

C. To 3 ml of solution containing about 0.1 mg of Iron or to 3 ml of the prescribed solution add 1 ml of *2 M hydrochloric acid* and 1 ml of *ammonium thiocyanate solution;* the solution becomes blood-red in colour. Take two portions, each of 1 ml, of the mixture. To one portion add 5 ml of *ether,* shake and allow to stand; the ether layer is pink. To the other portion add 3 ml of *0.2 M mercuric chloride;* the red colour disappears.

Ferrous Salts

A. To 1 ml of a solution containing not less than 1 mg of iron or to 1 ml of the prescribed solution add 1 ml of *potassium ferricyanide solution*; a dark blue precipitate is formed that is insoluble in *dilute hydrochloric acid* and is decomposed by *sodium hydroxide solution.*

B. To 2 ml of solution containing about 0.1 mg of iron or to 3 ml of the prescribed solution add *1M sodium hydroxide*; a greenish white precipitate is formed; when shaken, the colour change rapidly to green and then to brown.

Iodides

A. Dissolve a quantity of the substance under examination containing 4 mg of iodide ion in 2 ml of *water* or use 2 ml of the prescribed solution. Acidify with *dilute nitric acid* and add 0.5 ml of *silver nitrate solution.* Shake and allow to stand; a curdy, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 ml, of *water.* Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 ml of *water* and add 1.5 ml of *10 M ammonia;* the precipitate does not dissolve.

B. To 0.2 ml of solution of the substance under examination containing 5 mg of iodide ion per ml or to 0.2 ml of the prescribed solution add 0.5 ml of *dilute sulphuric acid,* 0.1 ml of *potassium dichromate solution,* 2 ml of *water* and 2 ml of *chloroform* shake for few seconds and allow to stand; the chloroform layer is violet or violet-red.

Lactates

To 5 ml of a solution of the substance under examination containing about 5 mg of *lactic acid* or to 5 ml of the prescribed solution add 1 ml of *bromine water* and 0.5 ml of *1 M sulphuric acid*. Heat on a water-bath, stirring occasionally with a glass rod until the colour is discharged. Add 4 g of *ammonium sulphate*, mix and add dropwise, without mixing, 0.2 ml of a 10 per cent w/v solution of *sodium nitroprusside* in *1 M*

sulphuric acid. Without mixing, add 1 ml of *strong ammonia solution* and allow to stand for 30 minutes; a dark green ring appears at the interface of the two liquids.

Lead Compounds

A. Dissolve 0.1 g of the substance under examination in 1 ml of *dilute acetic acid* or use 1 ml of the prescribed solution. Add 2 ml of *potassium chromate solution*; a yellow precipitate insoluble in 2 ml of *10 M sodium hydroxide* is produced.

B. Dissolve 50 mg of the substance under examination in 1 ml of *dilute acetic acid* or use 1 ml of the prescribed solution. Add 10 ml of *water* and 0.2 ml of *1 M potassium iodide*; a yellow precipitate is formed. Heat to boiling for 1 or 2 minutes and allow to cool; the precipitate is reformed as glistening, yellow plates.

Magnesium Salts

A. Dissolve about 15 mg of the substance under examination in 2 ml of *water* or use 2 ml of the prescribed solution. Add 1 ml of *dilute ammonia solution*; a white precipitate forms that is redissolved by adding 1 ml of *2 M ammonium chloride*. Add 1 ml of *0.25 M disodium hydrogen phosphate*; a white crystalline precipitate is produced.

B. To 0.5 ml of a neutral or slightly acid solution of the substance under examination add 0.2 ml of a 0.1 per cent w/v solution of *titan yellow* and 0.5 ml of *0.1 M sodium hydroxide*; a bright red turbidity develops which gradually settles to give a bright red precipitate.

Mercury Compounds

A. Place a solution of the substance under examination, free from an excess of *nitric acid*, on a well-scraped bright copper foil; stain produced becomes bright and silvery on rubbing.

B. To a solution of the substance under examination with *hydrogen sulphide solution*, yield a black precipitate that is insoluble in *ammonium sulphide solution* and in boiling *2 M nitric acid*.

Mercuric Salts

A. To the prescribed solution add *2 M sodium hydroxide* until strongly alkaline; a dense, yellow precipitate is produced.

B. To the prescribed solution of the substance under examination add carefully *potassium iodide solution*; a red precipitate is produced which is very soluble in an excess of the reagent.

Mercurous Salts

A. To a solution of the substance are decomposed by *1 M sodium hydroxide*, a black color is produced.

B. To a solution of the substance under examination add *hydrochloric acid*; a white precipitate is produced, which is blackened by adding *6M ammonia hydroxide*.

C. To a solution of the substance under examination add carefully *potassium iodide solution*; a yellow precipitate which may become green on standing, is produced.

Nitrates

A. Dissolve 15 mg of the substance under examination in 0.5 ml of *water*, add cautiously 1 ml of *sulphuric acid*, mix and cool. Incline the tube and carefully add, without mixing, 0.5 ml of *ferrous sulphate solution*; a brown colour is produced at the interface of the two liquids.

B. To a mixture of 0.1 ml of *nitrobenzene* and 0.2 ml of *sulphuric acid* add a quantity of the powdered substance under examination equivalent to about 1 mg of nitrate ion or the prescribed quantity. Allow to stand for 5 minutes and cool in ice whilst adding slowly with stirring 5 ml of *water* and then 5 ml of *sodium hydroxide solution*. Add 5 ml of *acetone*, shake and allow to stand; the upper layer shows an intense violet colour.

Nitrites

When treated with dilute *mineral acids* or with *6 M acetic acid*, nitrites evolve brownish-red fumes. The solution colours starch-iodide paper blue.

Penicillins

To 2 mg of the substance under examination add 2 mg of *chromotropic acid sodium salt* and 2 ml *sulphuric acid* and immerse in an oil-bath at 150°; the solution, when shaken and examined every 30 seconds, exhibits the colours stated in Table 1.

Penicillins and Cephalosporins

Carry out Tests A and B unless otherwise stated in the monograph.

A. Place 2 mg of the substance under examination in a test-tube (about 15 cm x 15 mm), moisten with 0.05 ml of *water* and add 2 ml of *sulphuric acid (95 per cent)*. Mix the contents of the tube by swirling and examine the colour of the solution. Immerse the test-tube in a water-bath for 1 minute and examine the colour again. The solution exhibits the colours stated in columns 2 and 3 of Table 2.

B. Carry out the procedure described in Test A using 2 ml of a mixture of 2 ml of *formaldehyde solution* and 100 ml of *sulphuric acid (95 per cent)*. The solution exhibit the colours stated in columns 4 and 5 of Table 2.

Phosphates (Orthophosphates)

A. To 5 ml of the prescribed solution, neutralised to pH 7.0, add 5 ml of *silver nitrate solution*; a light yellow precipitate forms, the colour of which is not changed by boiling and which is readily soluble in *10 M ammonia* and in *dilute nitric acid*.

B. Mix 1 ml of the prescribed solution with 1 ml of *ammonical magnesium sulphate solution*; a white crystalline precipitate is formed.

C. To 2 ml of the prescribed solution and 2 ml of *dilute nitric acid* and 4 ml of *ammonium molybdate solution* and warm the solution; a bright yellow precipitate is formed.

Table 1

Time (min)	Ampicillin, Ampicillin Sodium, Ampicillin Trihydrate	Benzathine Penicillin, Benzylpenicillin Potassium/ Sodium	Carbenicillin Sodium	Cloxacillin Sodium	Phenoxymethyl-penicillin Potassium
0	Colourless	Yellow	Colourless	Colourless	Colourless
0.5	Colourless	Yellow	Light brown	Pale yellow	Colourless
1	Colourless	Yellow	Yellowish brown	Greenish yellow	Colourless
1.5	Colourless	Orange yellow	Greenish brown	Yellowish green	Pale pink
2	Purple	Orange yellow	Greenish brown	Green	Purple
2.5	Deep purple	Orange yellow	Brown	Greenish purple	Purple
3	Violet	Pale orange	Dark brown	Purple	Bluish violet
3.5	Violet	Orange or may char	Dark brown	Purple	Dark blue
4	Charred	–	–	–	–

Table 2

Substance	Sulphuric acid (95 per cent)	Sulphuric acid (95 per cent) after 1 minute at 100°	Formaldehyde solution and sulphuric acid (95 per cent)	Formaldehyde solution and sulphuric acid (95 per cent) after 1 minute at 100°
(1)	(2)	(3)	(4)	(5)
Amoxycillin Trihydrate			Almost colourless	Dark yellow
Ampicillin			Almost colourless	Dark yellow
Ampicillin Sodium			Almost colourless	Dark yellow
Ampicillin Trihydrate			Almost colourless	Dark yellow
Benzathine penicillin			Almost colourless	Reddish brown
Benzylpenicillin Potassium				Reddish brown
Benzylpenicillin Sodium			Almost colourless	Reddish brown
Carbenicillin Sodium			Almost colourless	Yellowish brown
Cephalexin	Almost colourless	Pale yellow	Pale yellow	Yellow
Cefadroxil			Yellow	Orange
Cephaloridine	Pale yellow	Almost colourless	Red	Brownish red
Cloxacillin Sodium			Slightly greenish yellow	Yellow
Phenoxymethyl penicillin Potassium			Reddish brown	Dark Reddish brown
Procaine Penicillin	Almost colourless	Almost colourless	Almost colourless	Reddish Brown

Potassium Salts

A. Dissolve about 0.1g of the substance under examination in 2 ml of *water* or use 2 ml of the prescribed solution. Add 1 ml of *dilute acetic acid* and 1 ml of a freshly prepared 10 per cent w/v solution of *sodium cobaltinitrite;* a yellow or orange yellow precipitate is produced immediately.

B. Dissolve 0.1 g of the substance under examination in 2 ml of *water* or use 2 ml of the prescribed solution. Heat the solution with 1 ml of *sodium carbonate solution;* no precipitate is formed. Add to the hot solution 0.05 ml of *sodium sulphide solution;* no precipitate is formed. Cool in ice, add 2 ml of a 15 per cent w/v solution of *tartaric acid* and allow to stand; a white, crystalline precipitate is produced.

Salicylates

A. To 1 ml of a 10 per cent w/v neutral solution add 0.5 ml of *ferric chloride solution;* a violet colour is produced which persists after the addition of 0.1 ml of *acetic acid.*

B. Dissolve 0.5 g of the substance under examination in 10 ml of *water* or use 10 ml of the prescribed solution. Add 0.5 ml of *hydrochloric acid;* the precipitate obtained after recrystallisation from hot *water* and drying at a pressure of 2 kPa melts at about 159° (2.4.21).

Silicates

In a lead or platinum crucible mix by means of a copper wire to obtain a thin slurry of the prescribed quantity of the substance under examination with 10 mg of *sodium fluoride* and a few drops of *sulphuric acid.* Cover the crucible with a thin transparent plate of plastic under which a drop of *water* is suspended and warm gently; within a short time a white ring is formed around the drop of water.

Silver Compounds

Dissolve 10 mg of the substance under examination in 10 ml of *water* or use 10 ml of the prescribed solution, when acidified with *hydrochloric acid* gives a curdy white precipitate, soluble in *dilute ammonia solution.*

Sodium Salts

A. Dissolve 0.1 g of the substance to be examined in 2 ml of *water* or use 2 ml of the prescribed solution. Add 2 ml of a 15 per cent w/v solution of *potassium carbonate* and heat to boiling. No precipitate is formed. Add 4 ml of *potassium pyroantimonate solution* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

B. Dissolve a quantity of the substance under examination equivalent to about 2 mg of sodium (Na^+) in 0.5 ml of *water* or use 0.5 ml of the prescribed solution. Add 1.5 ml of *methoxyphenylacetic acid reagent* and cool in ice-water for 30 minutes. A voluminous, white, crystalline precipitate is formed. Place in *water* at 20° and stir for 5 minutes. The precipitate does not disappear. Add 1 ml of *dilute ammonia.* The precipitate dissolves completely. Add 1 ml of *ammonium carbonate solution.* No precipitate is formed.

Sulphates

A. Dissolve about 50 mg of the substance under examination in 5 ml of *water* or use 5 ml of the prescribed solution. Add 1 ml of *dilute hydrochloric acid* and 1 ml of *barium chloride solution;* a white precipitate is formed.

B. Add 0.1 ml of *iodine solution* to the suspension obtained in test A: the suspension remains yellow (distinction from sulphites and dithionite) but is decolorized by adding, drop wise, *stannous chloride solution* (distinction from iodates). Boil the mixture; no coloured precipitate is formed (distinction from selenates and tungstates).

Sulphur in Organic Compounds

A. Burn about 20 mg of the substance under examination by the oxygen-flask method (2.3.34), using 15 ml of *water* and 2 ml of *hydrogen peroxide solution (10 vol)* as the absorbing liquid. When combustion is complete, boil the solution gently for 10 minutes, adding *water* if necessary, and cool. The resulting solution gives the reactions of sulphates.

B. To about 50 mg of the substance under examination add 0.25 g of *zinc metal and sodium carbonate reagent,* mix and transfer to a small, thin-walled test-tube of hard glass and cover with a layer of the reagent. Carefully heat the tube to red heat, starting at the upper end and heating towards the bottom, and then drop the tube immediately into about 20 ml of *water.* Filter and acidify the filtrate with *hydrochloric acid;* fumes which stain *lead acetate paper* brown or black are evolved.

Tartrates

A. Dissolve about 20 mg of the substance under examination in 5 ml of *water* or use 5 ml of the prescribed solution. Add 0.05 ml of a 1 per cent w/v solution *of ferrous sulphate* and 0.05 ml of *hydrogen peroxide solution (10 vol);* a transient yellow colour is produced. After the colour has disappeared add *dilute sodium hydroxide solution* dropwise; an intense violet or purple colour is produced.

B. Heat 0.1 ml of 1.5 per cent w/v solution of *tartaric acid* or to 0.1 ml of prescribed solution add 0.1 ml of a 10 per cent w/v solution of *potassium bromide,* 0.1 ml of 2 per cent w/v solution of *resorcinol* and 3 ml of *sulphuric acid.* Heat on water-bath for 5 minutes. A dark blue colour that changes to red when the solution is cooled and poured into *water* is produced.

Thiosulphates

A. Dissolve 0.1 g of the substance under examination in 5 ml of *water* and add 2 ml of *hydrochloric acid;* a white precipitate is formed which soon turns yellow and sulphur dioxide, recognizable by its odour, is evolved.

B. Dissolve 0.1 g of the substance under examination in 5 ml of *water* and add 2 ml of *ferric chloride test solution;* a dark violet colour which quickly disappears, is produced.

Xanthines

Mix a few mg of the substance under examination or the prescribed quantity with 0.1 ml of *hydrogen peroxide solution (100 vol)* and 0.3 ml of *2 M hydrochloric acid,* heat to dryness on a water-bath until a yellowish red residue is produced and add 0.1 ml of *2 M ammonia;* the colour of the residue changes to reddish violet.

Zinc Salts

Dissolve 0.1 g of the substance under examination in 5 ml of *water* or use 5 ml of the prescribed solution. Add 0.2 ml of *strong sodium hydroxide solution;* a white precipitate is produced. Add a further 2 ml of *strong sodium hydroxide solution:* the precipitate dissolves. Add 10 ml of *ammonium chloride solution;* the solution remains clear. Add 0.1 ml of *sodium sulphide solution;* a flocculent, white precipitate is produced.

2.3.2. Identification of Barbiturates

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. Shake a mixture of 80 volumes of *chloroform,* 15 volumes of *ethanol (95 per cent)* and 5 volumes of *strong ammonia solution.* Use the lower layer.

Test solution. A 0.1 per cent w/v solution of the substance under examination in *ethanol (95 per cent).*

Reference solution. A 0.1 per cent w/v solution of the corresponding *Reference Substance* in *ethanol (95 per cent).*

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 18 cm. Remove the plate, allow to dry and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

2.3.3. Identification of Phenothiazines

Determine protected from light by thin-layer chromatography (2.4.17), coating the plate with *kieselguhr G.* Place the dry plate in a tank containing a shallow layer of a mixture of 85 volumes of *acetone,* 10 volumes of *2-phenoxyethanol* and 5 volumes of *polyethylene glycol 300* so that the plate dips about 5 mm beneath the surface of the liquid. Allow the solvent to rise 18 cm above the line of application. Remove the plate from the tank and use it immediately.

Mobile phase. Shake a mixture of 100 volumes of *light petroleum (40° to 60°)* and 2 volumes of *diethylamine* with 6 to 8 volumes of *2-phenoxyethanol* until a persistent cloudiness is obtained, decant and use the supernatant layer.

Test solution. A 0.2 per cent w/v solution of the substance under examination in *chloroform.*

Reference solution. A 0.2 per cent w/v solution of the corresponding *Reference Substance.*

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air, and examine in ultraviolet light at 365 nm and observe the fluorescence produced after a few minutes. Spray the plate with *ethanolic sulphuric acid (10 per cent v/v)* and observe the colour produced. The principal spot in the chromatogram obtained with the test solution corresponds in position, fluorescence and colour to that in the chromatogram obtained with the reference solution and has a similar stability for at least 20 minutes after spraying.

Ignore any spot remaining on the line of application. Unless otherwise specified, any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

2.3.4. Related Substances in Barbiturates

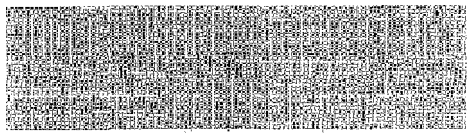
Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. Shake a mixture of 80 volumes of *chloroform,* 15 volumes of *ethanol (95 per cent)* and 5 volumes of *strong ammonia solution.* Use the lower layer.

Test solution. A 1.0 per cent w/v solution of the substance under examination in *ethanol (95 per cent).*

Reference solution. A 0.005 per cent w/v solution of the substance under examination in *ethanol (95 per cent).*

Apply to the plate 20 µl of each solution. After development, examine the plate immediately in ultraviolet light at 254 nm. Spray the plate with *diphenylcarbazone mercuric reagent,* allow it to dry in air and spray with a mixture of 1 volume of freshly prepared *ethanolic potassium hydroxide solution* and 4 volumes of *aldehyde-free ethanol (95 per cent).* Heat at 100° to 105° for 5 minutes and examine it immediately. By both methods of visualisation, any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution. Ignore any spot remaining on the line of application.



2.3.5. Related Substances in Phenothiazines

Determine protected from light in an atmosphere of nitrogen by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase (a). A mixture of 80 volumes of *cyclohexane*, 10 volumes of *acetone* and 10 volumes of *diethylamine*.

Mobile phase (b). A mixture of 85 volumes of *hexane*, 10 volumes of *acetone* and 5 volumes of *diethylamine*.

Mobile phase (c). A mixture of 90 volumes of *1-butanol* and 18 volumes of *1 M ammonia*.

Test solution. A freshly prepared 2.0 per cent w/v solution of the substance under examination in a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine*.

Reference solution. A freshly prepared 0.010 per cent w/v solution of the substance under examination in a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air, and examine in ultraviolet light at 254 nm. Ignore any spot remaining on the line of application. Unless otherwise specified, any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

2.3.6. Related Foreign Steroids

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase (a). A mixture of 77 volumes of *dichloromethane*, 15 volumes of *ether*, 8 volumes of *methanol* and 1.2 volumes of *water*.

Mobile phase (b). A mixture of 95 volumes of *1,2-dichloroethane*, 5 volumes of *methanol* and 0.2 volume of *water*.

Test solution. A 1.5 per cent w/v solution of the substance under examination in a mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*.

Reference solution (a) A 1.5 per cent w/v solution of the corresponding *Reference Substance* in the same solvent mixture.

Reference solution (b). A solution containing 0.03 per cent w/v each of *prednisolone RS, prednisone RS* and *cortisone RS* in the same solvent mixture.

Reference solution (c). A solution containing 0.03 per cent w/v each of *prednisolone acetate RS, prednisone RS, cortisone RS*, and *desoxycortone acetate RS* in the same solvent mixture.

Apply to the plate 1 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 105° for 10 minutes, cool and spray with *alkaline blue tetrazolium solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). Any secondary spot in the chromatogram obtained with the test solution is not more intense than the proximate spot in the chromatogram obtained with reference solution (b) or reference solution (c).

2.3.7. Related Substances in Sulphonamides

Method A

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 50 volumes of *1-butanol* and 10 volumes of *1 M ammonia*.

Test solution. Dissolve 1.0 g of the substance under examination in sufficient of a mixture of 90 volumes of *ethanol (95 per cent)* and 10 volumes of *strong ammonia solution* to produce 100 ml.

Reference solution. A 0.0050 per cent w/v solution of *sulphanilamide* in the same solvent mixture.

Apply to the plate 10 µl of each solution. After development, dry the plate, heat it at 105° for 10 minutes and spray with a 0.1 per cent v/v solution of *4-dimethylaminobenzaldehyde* in *ethanol (95 per cent)* containing 1 per cent v/v of *hydrochloric acid*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Method B

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 80 volumes of *chloroform*, 8 volumes of *methanol* and 4 volumes of *dimethylformamide*.

Test solution. Dissolve 0.25 g of the substance under examination in sufficient of a mixture of 90 volumes of *ethanol (95 per cent)* and 10 volumes of *strong ammonia solution* to produce 100 ml.

Reference solution. A 0.00125 per cent w/v solution of *sulphanilamide* in the same solvent mixture.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and spray with *ethanolic sulphuric acid (10 per cent v/v)*, heat at 105° for 30 minutes and immediately expose to nitrous fumes in a closed glass tank for 15 minutes. (Nitrous fumes may be generated by adding *sulphuric acid*

(50 per cent v/v) dropwise to a solution containing 10 per cent w/v of *sodium nitrite* and 3 per cent w/v of *potassium iodide*). Place the plate in a current of warm air for 15 minutes and spray with a 0.5 per cent w/v solution of *N- (1-naphthyl) ethylenediamine dihydrochloride* in *ethanol (95 per cent)*. If necessary, allow to dry and repeat the spraying. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Method C

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *dioxan*, 40 volumes of *nitromethane*, 5 volumes of *water* and 3 volumes of *6 M ammonia*.

Test solution. Dissolve 0.1 g of the substance under examination in 0.5 ml of *strong ammonia solution* and dilute to 5 ml with *methanol*; if the solution is not clear, heat gently until dissolution is complete.

Reference solution (a). A 0.40 per cent w/v solution of the substance under examination in a mixture of 24 volumes of *methanol* and 1 volume of *strong ammonia solution*.

Reference solution (b). A 0.010 per cent w/v solution of the substance under examination in the same solvent mixture.

Reference solution (c). A 0.40 per cent w/v solution of the corresponding *Reference Substance* in the same solvent mixture.

Apply to the plate 5 µl of each solution. After development, dry the plate at 100° to 105° and examine in ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b).

Limit Tests

2.3.8. Aluminium

Place the test solution in a separating funnel and shake with 2 quantities, each of 20 ml, and then with one 10 ml quantity of a 0.5 per w/v solution of *hydroxyquinoline* in *chloroform*. Dilute the combined chloroform solutions to 50.0 ml with *chloroform*.

Prepare a standard solution in the same manner using the prescribed reference solution.

Prepare a blank in the same manner using the prescribed blank solution.

Measure the intensity of the fluorescence (2.4.5) of the test solution (f_1), of the standard solution (f_2) and of the blank (f_3), using an excitant beam at 392 nm and a secondary filter with a transmission band centred on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence of the test solution ($f_1 - f_3$) is not greater than that of the standard solution ($f_2 - f_3$).

2.3.9. Aluminium in Adsorbed Vaccines

Use Method A or B.

Method A

Shake the sample thoroughly and transfer a volume containing about 5 mg of aluminium to a 50 ml combustion flask. Add 1 ml of *sulphuric acid*, 0.1 ml of *nitric acid* and a few glass beads or a little pumice powder. Heat the solution until thick, white fumes are evolved. If any charring takes place add a few more drops of *nitric acid* and continue boiling until the solution is colourless. Allow to cool for a few minutes, carefully add 10 ml of *water* and boil until a clear solution is obtained. Allow to cool, add 0.05 ml of *methyl orange solution* and neutralize with *10 M sodium hydroxide*. If a precipitate forms, dissolve it by adding, dropwise, sufficient *1M sulphuric acid*. Transfer the solution to a 250-ml conical flask, rinsing the combustion flask with small quantities of *water* and adding to the main solution. Add 25.0 ml of *0.02 M disodium edeate*, 10 ml of *buffer solution pH 4.4* and boil gently for 3 minutes. Add 0.1 ml of *pyridylazonaphthol solution* and titrate the excess of disodium edeate in the hot solution with *0.02 M cupric sulphate* until the colour changes to purplish brown. Repeat the operation omitting the substance under examination.

1 ml of *0.02 M disodium edetate* is equivalent to 0.0005396 g of Al.

Method B

Shake the sample thoroughly and transfer 1 ml volume containing about 0.5 mg per ml to 1 mg per ml of aluminium to 250 ml conical flask. Take 1 ml of distilled water in another 250 ml conical flask as blank. To both flasks add 2 ml distilled water, 1 ml *sulphuric acid*, 0.1 ml of *nitric acid* and a few glass beads. Heat the solution till white fumes are evolved. Allow to cool for few minutes and add 10 ml of distilled water to obtain clear solution. Add 2 drops of *methyl orange* and neutralize with *10 M sodium hydroxide* till yellow color appears. Add 10 ml of *0.01 M EDTA*, 10 ml of *acetate buffer solution pH 4.4*. Boil gently and add 0.1 ml of *pyridiazonaphthol* solution. Titrate excess *disodium edetate* in the hot solution with *0.01M cupric sulphate* until the color changes to purple brown.

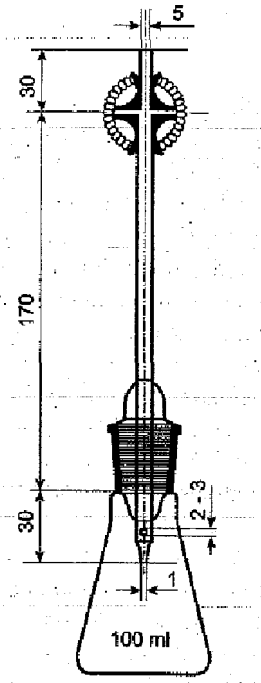
1 ml of *0.01M disodium edetate* is equivalent to 0.0002698 g of Al.

2.3.10. Arsenic

The limit for arsenic is indicated in the individual monographs in terms of ppm, i.e., the parts of arsenic, As, per million parts (by weight) of the substance under examination.

All reagents used for the test should have as low a content of arsenic as possible.

Apparatus



(Dimensions in mm)

Fig. 2.3.10-1: Apparatus for Limit Test For Arsenic

The apparatus (Fig. 2.3.10-1) consists of a 100-ml bottle or conical flask closed with a rubber or ground glass stopper through which passes a glass tube (about 20 cm x 5 mm). The lower part of the tube is drawn to an internal diameter of 1.0 mm, and 15 mm from its tip is a lateral orifice 2 to 3 mm in diameter. When the tube is in position in the stopper the lateral orifice should be at least 3 mm below the lower surface of the stopper. The upper end of the tube has a perfectly flat surface at right angles to the axis of the tube. A second glass tube of the same internal diameter and 30 mm long, with a similar flat surface, is placed in contact with the first and is held in position by two spiral springs or clips. Into the lower tube insert 50 to 60 mg of *lead acetate cotton*, loosely packed, or a small plug of cotton and a rolled piece of *lead acetate paper* weighing 50 to 60 mg. Between the flat surfaces of the tubes place a disc or a small square of *mercuric chloride paper* large enough to cover the orifice of the tube (15 mm x 15 mm).

Method

Into the bottle or conical flask introduce the test solution prepared as directed in the individual monograph, add 5 ml of *1 M potassium iodide* and 10 g of *zinc AsT*. Immediately assemble the apparatus and immerse the flask in a water-bath at a temperature such that a uniform evolution of gas is maintained. After 40 minutes any stain produced on the *mercuric chloride paper* is not more intense than that obtained by treating in the same manner 1.0 ml of *arsenic standard solution (10 ppm As)* diluted to 50 ml with *water*.

2.3.11. Calcium in Adsorbed Vaccines

Determine the calcium by atomic emission spectrometry (2.4.3). Homogenise the preparation under examination. To 1.0 ml add 0.2 ml of *dilute hydrochloric acid* and dilute to 3.0 ml with *distilled water*. Measure the absorbance at 620 nm.

2.3.12. Chlorides

Dissolve the specified quantity of the substance under examination in *water*, or prepare a solution as directed in the individual monograph and transfer to a Nessler cylinder. Add 10 ml of *dilute nitric acid*, except when nitric acid is used in the preparation of the solution, dilute to 50 ml with *water* and add 1 ml of *0.1 M silver nitrate*. Stir immediately with a glass rod and allow to stand for 5 minutes protected from light. When viewed transversely against a black background any opalescence produced is not more intense than that obtained by treating a mixture of 10.0 ml of *chloride standard solution (25 ppm Cl)* and 5 ml of *water* in the same manner.

2.3.13. Heavy Metals

The limit for heavy metals is indicated in the individual monographs in terms of ppm, i.e., the parts of lead, Pb, per million parts (by weight) of the substance under examination.

Method A

Buffer solution. Dissolve 25 g of *ammonium acetate* in 25 ml of *water*, add 38 ml of *6 M hydrochloric acid*. Adjust, if necessary with *3 M ammonia* or *6 M hydrochloric acid* to pH 3.5 and dilute with *water* to 100 ml and mix.

Standard solution. Into a 50-ml Nessler cylinder pipette 1.0 ml of *lead standard solution (20 ppm Pb)* and dilute with *water* to 25 ml. Adjust with *dilute acetic acid* or *dilute ammonia solution* to a pH between 3.0 and 4.0, dilute with *water* to about 35 ml and mix.

Test solution. Into a 50-ml Nessler cylinder place 25 ml of the solution prepared for the test as directed in the individual

monograph or dissolve the specified quantity of the substance under examination in sufficient *water* to produce 25 ml. Adjust with *dilute acetic acid* or *dilute ammonia solution* to a pH between 3.0 and 4.0, dilute with *water* to about 35 ml and mix.

Procedure. To each of the cylinder containing the standard solution and the test solution respectively, add 2 ml of buffer solution and 1.2 ml of *thioacetamide reagent*, dilute to 50 ml with *water*, mix, allow to stand for 5 minutes and view downwards over a white surface; the colour produced with the test solution is not more intense than that produced with the standard solution.

Method B

Buffer solution. Proceed as directed under Method A.

Standard solution. Proceed as directed under Method A.

Test solution. Weigh in a suitable crucible the quantity of the substance specified in the individual monograph, add sufficient *sulphuric acid* to wet the sample, ignite carefully at a low temperature until thoroughly charred. Add to the charred mass 2 ml of *nitric acid* and 5 drops of *sulphuric acid* and heat cautiously until white fumes are no longer evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burnt off. Cool, add 4 ml of *hydrochloric acid*, cover, digest on a water-bath for 15 minutes, uncover and slowly evaporate to dryness on a water-bath. Moisten the residue with 1 drop of *hydrochloric acid*, add 10 ml of hot *water* and digest for 2 minutes. Add *ammonia solution* dropwise until the solution is just alkaline to *litmus paper*, dilute to 25 ml with *water* and adjust with *dilute acetic acid* to a pH between 3.0 and 4.0. Filter, if necessary, rinse the crucible and the filter with 10 ml of *water*, combine the filtrate and washings in a 50-ml Nessler cylinder, dilute with *water* to about 35 ml and mix.

Procedure: Proceed as directed under Method A.

Method C

Standard solution. Into a 50-ml Nessler cylinder pipette 1.0 ml of *lead standard solution (20 ppm Pb)*, add 5 ml of *dilute sodium hydroxide solution*, dilute with *water* to 50 ml and mix.

Test solution. Into a 50-ml Nessler cylinder place 25 ml of the solution prepared for the test as directed in the individual monograph, or dissolve the specified quantity of the substance under examination in a mixture of 20 ml of *water* and 5 ml of *dilute sodium hydroxide solution*. Dilute with *water* to 50 ml and mix.

Procedure. To each of the cylinders containing the standard solution and the test solution respectively add 5 drops of *sodium sulphide solution*, mix, allow to stand for 5 minutes and view downwards over a white surface; the colour produced with the test solution is not more intense than that produced with the standard solution.

Method D

Standard solution. Into a small Nessler cylinder pipette 10.0 ml of either *lead standard solution (1 ppm Pb)* or *lead standard solution (2 ppm Pb)*.

Test solution. Prepare as directed in the individual monograph and pipette 12 ml into a small Nessler cylinder.

Procedure. To the cylinder containing the standard solution add 2.0 ml of the test solution and mix. To each of the cylinders add 2 ml of *acetate buffer pH 3.5*, mix, add 1.2 ml of *thioacetamide reagent*, allow to stand for 2 minutes and view downwards over a white surface; the colour produced with the test solution is not more intense than that produced with the standard solution.

2.3.14. Iron

Dissolve the specified quantity of the substance under examination in *water*, or prepare a solution as directed in the monograph, and transfer to a Nessler cylinder. Add 2 ml of a 20 per cent w/v solution of *iron-free citric acid* and 0.1 ml of *thioglycollic acid*, mix, make alkaline with *iron-free ammonia solution*, dilute to 50 ml with *water* and allow to stand for 5 minutes. Any colour produced is not more intense than that obtained by treating in the same manner 2.0 ml of *iron standard solution (20 ppm Fe)* in place of the solution under examination.

2.3.15. Lead

The limit for lead is indicated in the individual monograph in terms of ppm, i.e., the parts of lead, Pb, per million parts (by weight) of the substance under examination.

The following method is based on the extraction of lead by solutions of dithizone.

All reagents used for the test should have as low a content of lead as practicable. All reagent solutions should be stored in containers of borosilicate glass. Glassware should be rinsed thoroughly with warm dilute nitric acid followed by water.

Method

Transfer the volume of the prepared sample directed in the monograph to a separator and, unless otherwise directed in monograph, add 6 ml of *ammonium citrate solution Sp* and 2 ml of *hydroxylamine hydrochloride solution Sp*. (For the determination of lead in iron salts use 10 ml of *ammonium citrate solution Sp*). Add two drops of *phenol red solution* and make the solution just alkaline (red in colour) by the addition of *strong ammonia solution*. Cool the solution if necessary and add 2 ml of *potassium cyanide solution Sp*. Immediately extract the solution with several quantities, each

of 5 ml, of *dithizone extraction solution*, draining off each extract into another separating funnel, until the dithizone extraction solution retains its green colour. Shake the combined dithizone solution for 30 seconds with 30 ml of a 1 per cent v/v solution of *nitric acid* and discard the chloroform layer. Add to the acid solution exactly 5 ml of *dithizone standard solution* and shake for 30 seconds; the colour of the chloroform layer is not more intense than that obtained by treating in the same manner a volume of *lead standard solution (1 ppm Pb)* equivalent to the amount of lead permitted in the substance under examination, in place of the solution under examination.

2.3.16. Potassium

To 10 ml of the prescribed solution add 2 ml of a freshly prepared 1 per cent w/v solution of *sodium tetraphenylborate*. Prepare a standard solution in the same manner using a mixture of *potassium standard solution (20 ppm K)* and 5 ml of *water*.

After 5 minutes, any opalescence in the test solution is not more intense than in the standard solution.

2.3.17. Sulphates

NOTE — The solutions used for this test should be prepared with distilled water.

To 1.0 ml of a 25.0 per cent w/v solution of *barium chloride* in a Nessler cylinder add 1.5 ml of *ethanolic sulphate standard solution (10 ppm SO_4)*, mix and allow to stand for 1 minute. Add 15 ml of the solution prepared as directed in the monograph or a solution of the specified quantity of the substance under examination in 15 ml of *water* and 0.15 ml of *5 M acetic acid*. Add sufficient *water* to produce 50 ml, stir immediately with a glass rod and allow to stand for 5 minutes. When viewed transversely against a black background any opalescence produced is not more intense than that obtained by treating in the same manner 15 ml of *sulphate standard solution (10 ppm SO_4)* in place of the solution under examination.

2.3.18. Sulphated Ash

Ignite a suitable crucible (for example, silica, platinum, porcelain or quartz) at 600 ± 50° for 30 minutes, allow to cool in a desiccator over silica gel or other suitable desiccant and weigh. Place the prescribed amount of the substance under examination in the crucible and weigh. Moisten the substance under examination with a small amount of sulfuric acid (usually 1 ml) and heat gently at as low a temperature as practicable until the sample is thoroughly charred. After cooling, moisten the residue with a small amount of sulfuric acid (usually 1 ml), heat gently until white fumes are no longer evolved and ignite at 600 ± 50° until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel or other suitable desiccant, weigh it again and calculate the percentage of residue.

If the amount of the residue so obtained exceeds the prescribed limit, repeat the moistening with sulfuric acid and ignition, as previously, for 30 minutes periods until 2 consecutive weighings do not differ by more than 0.5 mg or until the percentage of residue complies with the prescribed limit.

The amount of substance used for the test (usually 1-2 g) is chosen so that at the prescribed limit the mass of the residue (usually about 1 mg) can be measured with sufficient accuracy.

2.3.19. Total Ash

Method A. For crude vegetable drugs

Unless otherwise stated in the individual monograph, weigh accurately 2 to 3 g of the air-dried crude drug in a tared platinum or silica crucible and incinerate, gently at first, and gradually increase the temperature to 675 ± 25°, until free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot *water*, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness and heat the whole to a temperature of 675 ± 25°. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 ml of *ethanol (95 per cent)*, break up the ash with a glass rod, burn off the ethanol, and again heat the whole to a temperature of 675 ± 25°. Cool in a desiccator, weigh the ash, and calculate the percentage of ash with reference to the air-dried crude drug.

Method B. For all other substances

Heat a platinum or silica crucible to red heat for 30 minutes, allow to cool in a desiccator and weigh. Unless otherwise specified in the individual monograph, weigh accurately about 1 g of the substance under examination and evenly distribute it in the crucible. Dry at 100° to 105° for 1 hour and ignite to constant weight in a muffle furnace at 600° ± 25°. Allow the crucible to cool in a desiccator after each ignition. The material should not catch fire at any time during the procedure. If after prolonged ignition a carbon-free ash cannot be obtained proceed as directed in method A. Ignite to constant weight. Calculate the percentage of ash on the dried basis.

Acid-insoluble ash

Use Method C unless otherwise directed.

Method C. Boil the ash (Method A or B) with 25 ml of *2 M hydrochloric acid* for 5 minutes, collect the insoluble matter in a Gooch crucible or on an ashless filter paper, wash with hot *water*, ignite, cool in a desiccator and weigh. Calculate the percentage of acid-insoluble ash on the dried drug basis.

Method D. Place the ash (Method A or B), or the sulphated ash (2.3.18), as directed in the individual monograph, in a crucible, add 15 ml of *water* and 10 ml of *hydrochloric acid*, cover with a watch glass, boil for 10 minutes, and allow to cool. Collect the insoluble matter on an ashless filter paper, wash with hot *water* until the filtrate is neutral, ignite to dull redness, cool in an a desiccator and weigh. Calculate the percentage of acid-insoluble ash on the dried basis.

Water-soluble ash

Boil the ash (Method A or B) for 5 minutes with 25 ml of *water*, collect the insoluble matter in a Gooch crucible or an ashless filter paper, wash with hot *water*, and ignite for 15 minutes at a temperature not exceeding 450°. Subtract the weight of the insoluble matter from the weight of the ash; the difference in weight represents the water-soluble ash. Calculate the percentage of water-soluble ash on the dried basis.

2.3.20. Free Formaldehyde

Use Method A unless otherwise specified.

Method A

To 1.0 ml of a ten-fold dilution of the preparation under examination in a test-tube add 4.0 ml of *water* and 5.0 ml of *acetylacetone reagent*. Warm in a water-bath at 40° and allow to stand for 40 minutes. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 1.0 ml of a solution containing 0.002 per cent w/v of *formaldehyde*, CH_2O, in place of the dilution of the preparation. The comparison should be made examining the tubes down their vertical axes.

Method B

To 1.0 ml of ten-fold dilution of the preparation under examination in a test-tube add 2.0 ml of *water*, 1.0 ml of a 1 per cent w/v solution of *phenylhydrazine hydrochloride*, 0.5 ml of a 5 per cent w/v solution of *potassium ferricyanide* and 1.0 ml of *hydrochloric acid* and allow to stand for 15 minutes. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 1.0 ml of a solution containing 0.002 per cent w/v of *formaldehyde*, CH_2O, in place of the dilution of the preparation. The comparison should be made examining the tubes down their vertical axes.

2.3.21. *N, N*-Dimethylaniline

Determine by gas chromatography (2.4.13).

Method A

Internal standard solution. Unless otherwise specified in the individual monograph, dissolve 50 mg of *naphthalene* in sufficient *cyclohexane* to produce 50 ml. Dilute 5 ml of this solution to 100.0 ml with *cyclohexane*.

Test solution (a). Unless otherwise specified in the individual monograph, weigh accurately about 1.0 g of the substance under examination and transfer to a centrifuge tube, add 5 ml of *1 M sodium hydroxide*, swirl to dissolve the sample, add 1.0 ml of *cyclohexane*, shake vigorously for 1 minute, and centrifuge. Use the upper layer.

Test solution (b). Unless otherwise specified in the individual monograph, weigh accurately about 1.0 g of the substance under examination and transfer to a centrifuge tube, add 5 ml of *1 M sodium hydroxide*, swirl to dissolve the sample, add 1.0 ml of the internal standard solution, shake vigorously for 1 minute, and centrifuge. Use the upper layer.

Reference solution. Unless otherwise specified in the individual monograph, weigh accurately about 50.0 mg of *N, N-dimethylaniline*, add 2 ml of *hydrochloric acid* and 20 ml of *water*, shake to dissolve and dilute to 50.0 ml with *water*. Dilute 5.0 ml of this solution to 250.0 ml with *water*. To 1.0 ml of the latter solution in a stoppered centrifuge tube add 5 ml of *1 M sodium hydroxide* and 1.0 ml of the internal standard solution. Stopper the tube and shake vigorously for 1 minute. Centrifuge if necessary and use the upper layer.

Chromatographic system
- a glass column 2 m x 2 mm, packed with silanised diatomaceous support (125 to 180 mesh) impregnated with 3 per cent w/w polymethylphenylsiloxane,
- temperature:
  column: 120°,
  inlet port and detector. 150°,
- flow rate: 30 ml per minute, using nitrogen as a carrier gas.

Inject 1 µl of each of the solutions. Record the chromatograms and measure the areas for the major peaks. In the chromatogram obtained with test solution (b) the ratio of the area of any peak due to *N, N*-dimethylaniline to the area of the peak due to the internal standard is not greater than the corresponding ratio in the chromatogram obtained with internal standard solution.

Method B

Internal standard solution. Unless otherwise stated in the individual monograph, dissolve 75 mg of *N, N-diethylaniline* in a mixture of 2 ml of *hydrochloric acid* and 20 ml of *water*

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Reference solu... erwise specified in the individual monograph, disso... mg of *N, N-dimethylaniline* in a mixture of 2 ml of *hydrochloric acid* and 20 ml of *water* and add sufficient *water* to produce 50 ml. Dilute 2 ml of the resulting solution to 100.0 ml with *water*. To 1 ml of the resulting solution add 1 ml of the internal standard solution, 1 ml of *dilute sodium hydroxide solution* and 1 ml of *cyclohexane*, shake vigorously for 1 minute, centrifuge if necessary and use the clear upper layer.

Chromatographic system

- a glass column 1.5 m x 4 mm, packed with 3 per cent w/w of cyanoethyl-silicone gum (such as XE-60) on *acid-washed silanised diatomaceous earth* (80 to 100 mesh),
- temperature:
  column: 80°,
  inlet port and detector: 150°,
- flow rate: 30 ml per minute, using nitrogen as a carrier gas.

Inject 1 µl of each of the solutions. Record the chromatograms and measure the areas for the major peaks. In the chromatogram obtained with test solution (b) the ratio of the area of any peak due to *N, N*-dimethylaniline to the area of the peak due to the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution.

Assays

2.3.22. Acetyl Value

The acetyl value is the number which expresses in milligrams the amount of potassium hydroxide required to neutralise the acetic acid liberated by the hydrolysis of 1 g of the acetylated substance.

Method

Determination the saponification value of the substance under examination (2.3.37).

Acetylate the substance under examination by the following method. Place 10 g with 20 ml of *acetic anhydride* in a long-necked, round-bottomed 200-ml flask attached to a reflux air condenser. Support the flask on a sheet of heat-resistant material in which a hole of about 4 cm in diameter has been cut and heat it with a small, naked flame, not more than 25 mm in height and which does not impinge on the bottom of the flask. Boil gently for 2 hours, allow to cool, pour into 600 ml of *water* contained in a large beaker, add 0.2 g of *pumice powder* and boil for 30 minutes. Cool, transfer to a separator and discard the lower layer. Wash the acetylated product with three or more quantities, each of 50 ml, of a warmed saturated solution of *sodium chloride* until the washings are no longer acid to *litmus paper*. Finally shake with 20 ml of warm *water* and remove the aqueous layer as completely as possible. Pour the acetylated substance into a small dish, add 1 g of powdered *anhydrous sodium sulphate*, stir thoroughly and filter through a dry pleated filter. Determine the saponification value of the acetylated substance.

Calculate the Acetyl value from the expression:

$$\text{Acetyl value} = \frac{1335(b-a)}{(1335-a)}$$

where, a = saponification value of the substance;
b = saponification value of the acetylated substance.

2.3.23. Acid Value

The acid value is the number which expresses in milligrams the amount of potassium hydroxide necessary to neutralise the free acids present in 1 g of the substance.

Method

Unless otherwise specified in the individual monograph, dissolve about 10 g of the substance under examination, accurately weighed, in 50 ml of a mixture of equal volumes of *ethanol (95 per cent)* and *ether*, previously neutralised with *0.1 M potassium hydroxide* to *phenolphthalein solution*. If the sample does not dissolve in the cold solvent, connect the flask with a reflux condenser and warm slowly, with frequent shaking, until the sample dissolves. Add 1 ml of *phenolphthalein solution* and titrate with *0.1 M potassium hydroxide* until the solution remains faintly pink after shaking for 30 seconds. Calculate the acid value from the expression:

$$\text{Acid value} = \frac{5.61\,n}{w}$$

where, n = the number of ml of *0.1 M potassium hydroxide* required;
w = the weight, in g, of the substance.

NOTE — If the oil has been saturated with carbon dioxide for the purpose of preservation, gently reflux the solution of

the oil in ethanol (95 per cent) and ether for 10 minutes before titration. The oil may be freed from the carbon dioxide by exposing it in a shallow dish in a vacuum desiccator for 24 hours before weighing the sample.

2.3.24. Cineole

Weigh 3.0 g of the substance under examination, freshly dried over *anhydrous sodium sulphate*, into a dry test-tube and add 2.1 g of melted *o-cresol*. Place the tube in the apparatus for the freezing point (2.4.11), and allow to cool, stirring continuously. When crystallisation takes place there is a small rise in temperature; note the highest temperature reached (t_1).

Re-melt the mixture on a water-bath ensuring that the temperature does not exceed t_1 by more than 5° and place the tube in the apparatus maintained at a temperature 5° below t_1. When crystallisation takes place, or when the temperature of the mixture has fallen 3° below t_1, stir continuously; note the highest temperature at which the mixture freezes (t_2). Repeat the operation until the two highest values obtained for t_2 do not differ by more than 0.2°. If supercooling occurs, induce crystallisation by the addition of a small crystal of a complex consisting of 3.0 g of *cineole* and 2.1 g of melted *o-cresol*. If t_2 is below 27.4°, repeat the determination after the addition of 5.1 g of the complex.

Determine the percentage w/w of cineole corresponding to the freezing point (t_2) from Table, obtaining intermediate values by interpolation. If 5.1 g of the cineole-*o*-cresol complex was added, calculate the percentage w/w of cineole from the expression 2(A-50), where A is the value corresponding to a freezing point of t_2 taken from the table (see below).

Table

t_2°	Cineole per cent w/w	t_2°	Cineole per cent w/w
24	45.5	40	67.0
25	47.0	41	68.5
26	48.5	42	70.0
27	49.5	43	72.5
28	50.5	44	74.0
29	52.0	45	76.0
30	53.5	46	78.0
31	54.5	47	80.0
32	56.0	48	82.0
33	57.0	49	84.0
34	58.5	50	86.0
35	60.0	51	88.5
36	61.0	52	91.0
37	62.5	53	93.5
38	63.5	54	96.0
39	65.0	55	99.0

2.3.25. Esters

Boil a convenient quantity of *ethanol (95 per cent)* thoroughly to expel carbon dioxide and neutralise it to *phenolphthalein solution*. Unless otherwise stated in the individual monograph, weigh accurately about 2 g or other suitable quantity of the substance under examination so that the volume of *0.5 M ethanolic potassium hydroxide* added is at least twice that theoretically required, dissolve it in 5 ml of the neutralised ethanol contained in a hard-glass flask and neutralise the free acid in the solution with *0.1 M ethanolic potassium hydroxide* using 0.2 ml of *phenolphthalein solution* as indicator. Add 25.0 ml of *0.5 M ethanolic potassium hydroxide* and boil under a reflux condenser on a water-bath for 1 hour. Add 20 ml of *water* and titrate the excess of alkali with *0.5 M hydrochloric acid* using a further 0.2 ml of *phenolphthalein solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the alkali required to saponify the esters.

2.3.26. Ester Value

The ester value is the number of milligrams of potassium hydroxide required to saponify the esters present in 1 g of the substance.

Determine the acid value (2.3.23), and the saponification value (2.3.37), of the substance under examination. Calculate the ester value from the expression:

Ester value = Saponification value - Acid value.

2.3.27. Hydroxyl Value

The hydroxyl value is the number of milligrams of potassium hydroxide required to neutralise the acid combined by acylation in 1 g of the substance.

Use method A unless otherwise specified.

Method A

Unless otherwise specified in the individual monograph, weigh accurately the quantity of the substance under examination, stated in the table (see below), in a 150-ml acetylation flask fitted with a condenser and add the quantity of *pyridine-acetic anhydride reagent* stated in the table. Boil for 1 hour on a water-bath, adjusting the level of the *water* to maintain it 2 to 3 cm above the level of the liquid in the flask all through. Cool, add 5 ml of *water* through the top of the condenser; if this causes cloudiness, add sufficient *pyridine* to produce a clear liquid. Shake; reflux in the water-bath for 10 minutes, remove and cool. Rinse the condenser and the walls of the flask with 5 ml of *ethanol (95 per cent)*, previously neutralised to *dilute phenolphthalein solution*. Titrate with

0.5 M ethanolic potassium hydroxide using *dilute phenolphthalein solution* as indicator. Perform a blank determination.

Table

Presumed hydroxyl value	Quantity of substance (g)	Volume of pyridine *acetic anhydride reagent (ml)*
10 to 100	2.0	5.0
101 to 150	1.5	5.0
151 to 200	1.0	5.0
201 to 250	0.75	5.0
251 to 300	0.60 or 1.20	5.0 or 10.0
301 to 350	1.00	10.0
351 to 700	0.75	15.0
701 to 950	0.5	15.0

Calculate the hydroxyl value from the expression:

$$\text{Hydroxyl value} = \text{Acid value} + \frac{28.05 \times v}{w}$$

where, v = difference, in ml, between the titrations;
w = weight, in g, of the substance.

Method B

Weigh accurately the specified quantity of the substance under examination into a flask fitted with a reflux condenser, add 12 g of *stearic anhydride* and 10 ml of *xylene* and heat under reflux for 30 minutes. Cool, add a mixture of 40 ml of *pyridine* and 4 ml of *water*, heat under reflux for a further 30 minutes and titrate the hot solution with *1 M potassium hydroxide* using *dilute phenolphthalein solution* as indicator. Perform a blank determination.

Calculate the hydroxyl value from the expression:

$$\text{Hydroxyl value} = \frac{56.11 \times v}{w}$$

where, v = difference, in ml, between the titrations;
w = weight, in g, of the substance.

2.3.28. Iodine Value

The iodine value is the number which expresses in grams the quantity of halogen, calculated as iodine, which is absorbed by 100 g of the substance under the described conditions. It may be determined by any of the following methods.

Method A (Iodine Monochloride Method or Wijs Method)

Place an accurately weighed quantity of the substance under examination in a dry 500-ml iodine flask, add 10 ml of *carbon tetrachloride* and dissolve. Add 20 ml of *iodine monochloride solution*, insert the stopper and allow to stand in the dark at a temperature between 15° and 25° for 30 minutes. Place 15 ml of *potassium iodide solution* in the cup top, carefully remove the stopper, rinse the stopper and the sides of the flask with 100 ml of *water*, shake and titrate with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Note the number of ml required (a). Repeat the operation without the substance under examination and note the number of ml required (b).

Calculate the iodine value from the expression:

$$\text{Iodine value} = \frac{1.269(b-a)}{w}$$

where, w = weight, in g, of the substance.

The approximate weight, in g, of the substance to be taken may be calculated by dividing 20 by the highest expected iodine value. If more than half the available halogen is absorbed, the test must be repeated with a smaller quantity of the substance.

Method B (Iodine Monobromide Method or Hanus Method)

Unless otherwise specified, weigh accurately the quantity of the substance under examination, stated in the table (see below), place it in a dry 300-ml iodine flask or which has been rinsed with *glacial acetic acid* unless otherwise specified in the monograph. Add 15 ml of *chloroform* and dissolve. Add slowly from a burette 25.0 ml of *iodine monobromide solution*, insert the stopper, allow to stand in the dark for 30 minutes, unless otherwise specified in the monograph, shaking frequently. Add 10 ml of *potassium iodide solution* and 100 ml of *water* and titrate with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Note the number of ml required (a). Repeat the operation without the substance under examination and note the number of ml required (b). Calculate the iodine value from the expression given under Method A.

The approximate weight, in g, of the substance to be taken, unless otherwise specified in the monograph, may be calculated from the table.

Table

Presumed Iodine Value	Quantity of the substance (g)
Less than 20	1.0
20 to 60	0.25 to 0.5
61 to 100	0.15 to 0.25
More than 100	0.10 to 0.15

Method C (Pyridine Bromide Method)

Place an accurately weighed quantity of the substance under examination in a dry iodine flask, add 10 ml of *carbon tetrachloride* and dissolve. Add 25 ml of *pyridine bromide*

solution, allow to stand for 10 minutes in the dark and complete the determination described under Method A beginning at the words "Place 15 ml of...".

The approximate weight, in g, of the substance to be taken may be calculated by dividing 12.5 by the highest expected iodine value. If more than half the available halogen is absorbed, the test must be repeated with a smaller quantity of the substance.

2.3.29. Methoxyl

Apparatus

The apparatus consists of a 50-ml round-bottomed boiling flask into which is sealed a capillary side arm of 1 mm diameter to provide an inlet for a stream of *carbon dioxide* or *nitrogen*. The flask is also fitted with an upright air condenser about 25 cm long and about 9 mm in diameter, bent through 180° at the top and terminating in a glass capillary of 2 mm diameter dipping into a small scrubber containing about 2 ml of *water*. The outlet from the scrubber is a tube of about 7 mm diameter which dips below the surface of the liquid in the first of two receivers connected in series.

Method

Weigh accurately a quantity of the substance under examination containing approximately 50 mg of methyl iodide and place it in the boiling flask. Add a little pumice, 2.5 ml of melted *phenol* and 5 ml of *hydriodic acid* and connect the flask with the remainder of the apparatus. The first receiver contains about 6 ml and the second receiver about 4 ml of a 10 per cent w/v solution of *potassium acetate* in *glacial acetic acid* to which 0.2 ml of *bromine* has been added. Pass a slow uniform stream of *carbon dioxide* or *nitrogen* through the side arm of the boiling flask and gently heat the liquid by means of a mantled micro-burner at such a rate that the vapours of the boiling liquid rise half-way up the condenser. For most substances 30 minutes is sufficient to complete the reaction and sweep out the apparatus. Wash the contents of both receivers into a 250-ml glass-stoppered conical flask containing 5 ml of a 25 per cent w/v solution of *sodium acetate*, adjust the volume of the liquid to approximately 125 ml and add 0.3 ml of *formic acid*. Rotate the flask until the colour due to the bromine is discharged, add 0.6 ml of *formic acid*, stopper the flask and mix the contents thoroughly so as to remove any excess of bromine from the vapour above the liquid in the flask. After allowing to stand for 1 to 2 minutes, add 1 g of *potassium iodide* and a few ml of *1 M sulphuric acid* and titrate the liberated iodine with *0.1 M sodium thiosulphate*. Perform a blank titration and make any necessary correction.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.0005172 g of methoxyl (CH_3O).

2.3.30. Nitrogen

Use method E for substances containing 2 mg or less of nitrogen.

Method A

Weigh accurately the quantity of the substance under examination specified in the individual monograph or a quantity equivalent to about 35 mg of nitrogen into a 200-ml long-necked flask and add 3 g of *anhydrous sodium sulphate*, 0.3 g of *nitrogen-free mercuric oxide* and 20 ml of *nitrogen-free sulphuric acid*, unless otherwise specified in the monograph. Heat the mixture over a small flame until colourless and boil gently for a further 2 hours, unless otherwise directed in the monograph, care being taken to prevent the upper part of the flask from getting overheated. Cool, cautiously dilute to about 75 ml with *water* and add a piece of *granulated zinc* and a solution containing 1.5 g of *sodium hydroxide* per ml of the sulphuric acid used and 2 g of *sodium thiosulphate* in 25 ml of *water*. Ensure that before distillation the mixture is strongly alkaline by increasing, if necessary, the quantity of *sodium hydroxide*. Immediately connect the flask to a distillation apparatus, mix the contents, distil the liberated ammonia into 50.0 ml of *0.1 M sulphuric acid* and titrate the excess of acid with *0.1 M sodium hydroxide* using *methyl red-methylene blue* solution as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the ammonia liberated by the substance under examination.

1 ml of *0.1 M sulphuric acid* is equivalent to 0.002802 g of N.

Method B

Weigh accurately the quantity of the substance under examination specified in the monograph or a quantity equivalent to about 35 mg of nitrogen into a 200-ml long-necked flask, add 20 ml of *nitrogen-free sulphuric acid*, unless otherwise specified in the monograph, and heat for 15 minutes. Add 3 g of *anhydrous sodium sulphate* and 0.3 g of *nitrogen-free mercuric oxide* and complete Method A, beginning at the words "Heat the mixture...".

1 ml of *0.1 M sulphuric acid* is equivalent to 0.002802 g of N.

Method C

Weigh accurately the quantity of the substance under examination specified in the monograph or a quantity equivalent to about 15 mg of nitrogen into a 200-ml long-necked flask and add 1 g of a powdered mixture of 10 parts of *anhydrous sodium sulphate* or *potassium sulphate* and 1 part of *cupric sulphate*. Add 10 ml of *nitrogen-free sulphuric acid*, mix, and carefully add 1 ml of *hydrogen peroxide solution*

(100 vol) carefully down the wall of the flask. Heat until the solution becomes clear green in colour or almost colourless for 30 minutes. Cool, carefully add 20 ml of *water*, cool again and connect the flask to a distillation apparatus. Add 50 ml of *10 M sodium hydroxide* and distil immediately by passing steam through the flask. Collect the distillate in 25.0 ml of *0.1 M hydrochloric acid* and titrate the excess of acid with *0.1 M sodium hydroxide* using *methyl red-methylene blue solution* as indicator. Repeat the operation using 25 mg of *anhydrous dextrose* in place of the substance under examination. The difference between the titrations represents the ammonia liberated by the substance under examination.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.001401 g of N.

Method D *(When nitrates and nitrites are present)*

Weigh accurately the quantity of the substance under examination specified in the monograph or a quantity equivalent to about 15 mg of nitrogen into a 200-ml long-necked flask, add 10 ml of *nitrogen-free sulphuric acid* in which 0.2 g of *salicylic acid* has been previously dissolved and mix. Allow the mixture to stand for 30 minutes with frequent shaking and add 1 g of a powdered mixture of 10 parts of *anhydrous sodium sulphate* or *potassium sulphate* and 1 part of *cupric sulphate*, mix and carefully add 1 ml of *hydrogen peroxide solution (100 vol)* down the wall of the flask. Complete Method C beginning at the words "Heat until the solution...".

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.001401 g of N.

Method E

Apparatus: A unit of the type generally known as semi-micro Kjeldahl apparatus.

Method

Weigh accurately a quantity of the substance under examination equivalent to about 2 mg of nitrogen into the digestion flask of the apparatus. Add 1 g of a powdered mixture of 10 parts of *anhydrous sodium sulphate* or *potassium sulphate* and 1 part of *cupric sulphate* and wash down any adhering material from the neck of the flask with *water*. Add 7 ml of *nitrogen-free sulphuric acid* and 1 ml of *hydrogen peroxide solution (100 vol)* carefully down the wall of the flask. (Do not add hydrogen peroxide during the digestion). Heat until the solution has a clear blue colour and the sides of the flask are free from carbonaceous matter. Cool, add carefully 20 ml of *water*, cool the solution and arrange for steam distillation. Add through the funnel 30 ml of *10 M sodium hydroxide*, rinse the funnel with 10 ml of *water*, tightly close the apparatus and begin the distillation with steam immediately. Collect the distillate in 25.0 ml of *0.01 M sulphuric acid*, continue the distillation until the distillate measures about 100 ml. Titrate the distillate with *0.01 M sodium hydroxide* using *methyl red-methylene blue solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the ammonia liberated by the substance under examination.

1 ml of *0.01 M sulphuric acid* is equivalent to 0.0002802 g of N.

Method F *(Determination of Protein in Blood Products)*

For dried blood products prepare a solution of the preparation as directed in the monograph.

To a volume expected to contain about 0.1 g of protein add sufficient *saline solution* to produce 20 ml. To 2 ml of the resulting solution, in a 75-ml boiling tube, add 2 ml of a solution containing 75.0 per cent v/v of *nitrogen-free sulphuric acid*, 4.5 per cent w/v of *potassium sulphate* and 0.5 per cent w/v of *copper (II) sulphate*, mix and loosely stopper the tube. Heat gradually to boiling, boil vigorously for 1.5 hours and cool. If the solution is not clear add 0.25 ml of *hydrogen peroxide solution (20 vol)*, continue heating until a clear solution is produced and cool. During heating, take precautions to ensure that the upper part of the tube is not overheated.

Transfer the solution to a distillation apparatus using three 3-ml quantities of *water*, add 10 ml of *10 M sodium hydroxide* and distil rapidly for 4 minutes, collecting the distillate in a mixture of 5 ml of a saturated solution of *boric acid* and 5 ml of *water* and keeping the tip of the condenser below the level of the acid. Lower the collection flask so that the condenser can drain freely and continue the distillation for a further 1 minute. Titrate with *0.02 M hydrochloric acid* using *methyl red mixed solution* as indicator (V_1 ml).

To a further volume of the preparation under examination, or of the solution prepared from it, expected to contain about 0.1 g of protein, add 12 ml of *saline solution*, 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, allow to stand for 15 minutes, add sufficient *water* to produce 20 ml, shake again and centrifuge. Using 2 ml of the resulting clear supernatant liquid repeat the procedure described above beginning at the words 'in a 75-ml boiling tube...' (V_2 ml). Calculate the protein content in mg per ml of the preparation under examination, using the expression $6.25 \times 0.280 (V_1 - V_2)$ and taking into account the initial dilution.

Method G

Indicator solution. Dissolve 100 mg *methyl red* and 50 mg *methylene blue* in absolute ethanol and add sufficient quantity of absolute ethanol to produce 100 ml.

To 1 g of catalyst mixture of *copper sulphate* and *potassium sulphate* in the ratio 1:4 add substance under examination in volume equivalent to 40 mg of total protein or any suitable concentration derived during the validation studies in a Kjeldahl tube. Ensure that the sample is completely dispensed into the digestion tube and no amount is sticking on to the inner sides of the micro tips. Make up final volume to 1.0 ml in the respective tube with deionized water. Add suitable amount of *concentrated sulphuric acid* (5 ml) to the tube. For Blank, take 1 ml of distilled water instead of substance under examination.

NOTE — The ratio of copper sulphate and *potassium sulphate* in *catalyst mixture may be adjusted according to the sample concentration and validated accordingly.*

Digest the sample/ blank using digestion block at a temperature within 380° - 400° for 2 hours or till a clear solution is obtained.

Cool the digestion tubes and add 20 ml of deionized water. Add excess base (eg. 25 ml of *10 M sodium hydroxide*) to the acid digestion mixture and start distillation immediately. Depending on the volume of the digestion mixture and the method being followed, 15 to 150 ml of condensate should be collected in the receiving flask to ensure complete recovery of nitrogen. Collect the distillate in a receiver solution containing 1:1 ratio of saturated *boric acid* solution and deionized water and add 60 µl indicator solution.

Titrate the distillate with hydrochloric acid of appropriate normality (*0.02 M hydrochloric acid*) till the end point (from green to pink color) and record the volume of *hydrochloric acid* consumed. Determine the net volume of *hydrochloric acid* consumed by subtracting the volume of *hydrochloric acid* consumed by blank.

1 ml of *0.02 M hydrochloric acid* is equivalent to 0.2802 mg of nitrogen.

2.3.31. Nitrite Titration

The following method is suitable for the determination of most of the pharmacopoeial sulphonamide drugs and their preparations. It may also be used for other pharmacopoeial drugs for which nitrite titration is recommended.

Apparatus

A suitable open vessel of about 200 ml capacity is fitted with two similar clean platinum electrodes and a stirrer. The electrodes may be of platinum foil 0.5 cm square and should be placed 1.5 cm apart. They may be cleaned by immersing for a few seconds in boiling *nitric acid* containing a small amount of *ferric chloride*, followed by washing with *water*.

The polarising voltage may be obtained from a 1.5 volt dry cell and potentiometer or other convenient device which enables a small but definite voltage to be applied across the electrodes. The current flowing in the system is indicated by a series galvanometer which should have adequate sensitivity.

Method

Weigh accurately about 0.5 g in the case of a sulphonamide or otherwise the quantity specified in the individual monograph and transfer to the titration vessel. Add 20 ml of *hydrochloric acid* and 50 ml of *water* stir until dissolved, cool to about 15°. Immerse the platinum electrodes in the solution and apply a voltage of about 50 mV across the electrodes when polarisation of the electrodes takes place. Place the burette tip just above the surface of the solution and stir the solution gently, maintaining the temperature at about 15°. The titration may be carried out manually or by means of an automatic titrator. In the manual titration, add *0.1 M sodium nitrite* slowly and when the titration is within 1 ml of the end point, add the titrant in 0.1 ml portions, allowing not less than 1 minute between additions. (The galvanometer needle deflects and then returns to approximately its original position until the end point is reached). At the end-point, when a slight excess of sodium nitrite is present, the electrodes are depolarised, current flows and a permanent deflection of the needle is obtained.

The weight, in mg, of the substance to which each ml of *0.1 M sodium nitrite* is equivalent is as stated in the individual monograph.

For the assay of Tablets of the sulfonamides or other drugs, reduce not less than 20 tablets to a fine powder, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, and proceed as directed in the foregoing, beginning with "transfer to a suitable open vessel".

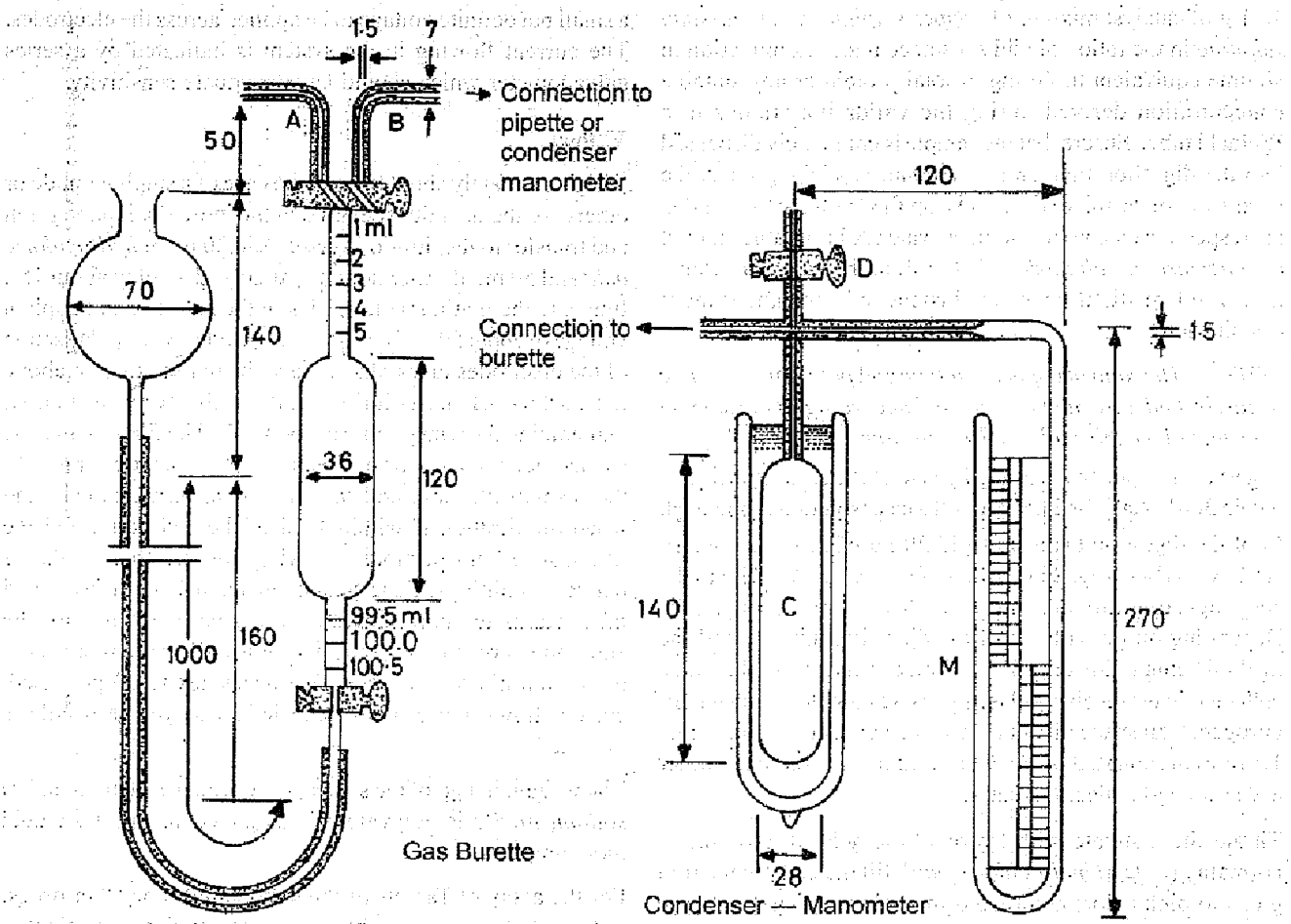
For the assay of Injections and other liquid forms where the nitrite titration is specified, pipet a portion, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, into a suitable open vessel, and proceed as directed in the foregoing, beginning with "Add 20 ml of *hydrochloric acid*".

NOTE—It will be necessary to adjust the sensitivity of the galvanometer or the applied voltage before the titration is begun in order to obtain an adequate deflection at the end-point.

2.3.32. Assay of Nitrous Oxide

Apparatus

The apparatus shown in Fig 2.3.32-1 comprises a gas burette of 100-ml capacity, connected through a two-way tap at its upper end to two capillaries, one of which (A) is used to introduce the gas into the apparatus, the other (B) being



(Dimensions in mm unless otherwise stated)

Fig. 2.3.32-1: Apparatus for Assay of Nitrous Oxide

connected to a vertical capillary arm to form a four-way junction. The descending arm of the junction is connected to a condenser (C) of about 60-ml capacity, and the right arm of the junction is connected to a mercury manometer (M). Tap D on the upper vertical arm of the junction opens to the air. The lower part of the gas burette is fitted with a one-way tap connected by a rubber tube to a mercury reservoir. The upper part of the burette is graduated from 0 to 5 ml, and the lower part from 99.5 to 100.5 ml, both in increments of 0.1 ml.

Method

Close the three taps and immerse the condenser in liquid nitrogen, keeping the level slightly above the upper part of the condenser. By manipulating the two-way tap and the mobile reservoir create a partial vacuum in the apparatus, choosing an arbitrary pressure, P_a, between 6.7 and 8 kPa (50 to 60 torr), accurately measured. This pressure must remain constant for 10 minutes to demonstrate that the apparatus is gas-tight.

Open the two-way tap to tube A and completely fill the burette and tube A with mercury. Close the two-way tap. Connect a rubber tube through a suitable pressure-relieving device to the exit valve of the cylinder of the gas under examination and pass a current of the gas through the rubber tube for 1 minute. Whilst the gas is still flowing, connect the rubber tube to the end of tube A and immediately open the two-way tap to tube A. Allow the specified volume of the gas to enter the burette by lowering the mercury reservoir. Disconnect the rubber tube and expel the gas from the burette by slowly raising the mercury reservoir above the capillary tube. Allow the specified quantity of the gas under examination to enter the burette by lowering the mercury reservoir and ensure that the pressure of the gas is equal to atmospheric pressure. Close the two-way tap.

Raise the mercury reservoir slightly above tube A and lower the level of the liquid nitrogen to the middle of the condenser. Carefully open the tap of the burette to connect with the condenser and allow the mercury to rise in the burette until it

reaches the tap. Close the tap. Raise the level of the liquid nitrogen so as to totally immerse the condenser. Read the pressure and wait until it remains steady for 2 minutes.

Place the mercury reservoir in its bottom position and open the tap of the burette to make connection with the condenser. Move the mercury reservoir until the manometer reading is the same as the initial pressure P_o. Close the tap of the burette and, by means of the mercury reservoir, bring the pressure of the gas in the burette to atmospheric pressure. The number of ml of gas represents the non-condensable volume in the specified volume used.

After each series of 10 determinations allow atmospheric air to enter by opening the tap D, remove the liquid nitrogen from the condenser and allow the condenser to warm to room temperature.

2.3.33. Assay of Oxygen

Apparatus

Oxygen in gases is determined using a paramagnetic analyzer.

The principle of the method is based on the high paramagnetic sensitivity of the oxygen molecule. Oxygen exerts a strong interaction on magnetic fields, which is measured electronically, amplified and converted to a reading of oxygen concentration. The measurement of oxygen concentration is dependent upon the pressure and temperature and, if the analyzer is not automatically compensated for variations in temperature and pressure, it must be calibrated immediately prior to use. As the paramagnetic effect of oxygen is linear, the instrument must have a suitable range with a readability of 0.1 per cent or better.

Method

Set the zero by passing *nitrogen* through the instrument until a constant reading is obtained. Set the scale to 100 per cent by passing *oxygen* through the instrument at the same flow rate as for *nitrogen* until a constant reading is obtained. Pass the gas under examination through the instrument at a constant flow rate until a constant reading is obtained. Record the concentration of oxygen in the gas under examination.

2.3.34. Oxygen-Flask Method

The oxygen flask combustion procedure is the preparatory step in the determination of bromine, chlorine, fluorine, iodine, selenium, and sulphur in some Pharmacopoeial substances. Combustion of the substance under examination (usually organic) yields water-soluble inorganic products, which are analyzed for specific elements as directed in this chapter or individual monograph.

NOTE— Great care must be taken in carrying out the following method. It is advisable to wear safety glasses and to use a suitable safety screen particularly when combustion takes place.

Apparatus

The apparatus (shown in Fig. 2.3.34-1) consists of a thick-walled, conical 500 ml iodine flask, fitted with a ground-glass stopper to which is fused a piece of platinum wire about 13 cm long and 1 mm in diameter and to which is attached a piece of platinum gauze to serve as means of holding the sample. The gauze may be about 2 cm wide and 1.5 cm long and should comply with the dimensions of a sieve with a nominal mesh aperture of 425 mm (No. 36 sieve). The flask must be well-cleaned and free from even traces of organic solvents.

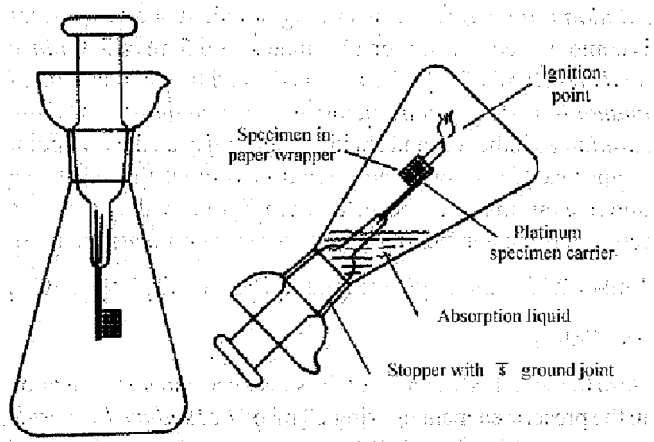


Fig 2.3.34 -1: Apparatus for Oxygen Flask Method

Method

Solid substances should be finely ground and thoroughly mixed before the specified quantity is weighed.

For liquids place the specified quantity on about 15 mg of ashless filter-paper flock contained in one part of a methylcellulose capsule of a suitable size, close the capsule, inserting one end of a narrow strip of filter-paper between the two parts, and secure the capsule in the platinum gauze.

Ointments should be enclosed in grease-proof paper before wrapping in filter-paper.

Weigh accurately a suitable quantity of the substance under examination and wrap, if a solid, in a piece of halide-free filter-paper (4 cm x 3 cm); secure the package in the platinum gauze sample holder and insert one end of a narrow strip (1 cm x 3 cm) of filter-paper in the roll to serve as a fuse. Flush the flask with *oxygen*, moisten the neck with *water*, place the specified absorbing liquid in the flask, fill it with *oxygen* by swirling the liquid to favour its taking up the oxygen, light the free end of the fuse-strip and immediately insert the stopper

Hold the stopper firmly in place when vigorous burning has begun, invert the flask so as to provide a liquid seal but taking care to prevent incompletely burned material falling into the liquid. When combustion is complete, shake the flask vigorously for about 5 minutes, place a few ml of *water* in the cup top, carefully remove the stopper, and rinse the stopper, platinum wire, platinum gauze, and sides of the flask with *water*. Proceed as directed in the following methods.

NOTE — Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.

For Bromine

Burn the specified quantity of the substance under examination in the prescribed manner using 15 ml of a mixture of 9 volumes of *0.5 M sulphuric acid* and 1 volume *hydrogen peroxide solution (100 vol)* as the absorbing liquid. When the process is complete, cool in ice for 15 minutes, add 5 ml of *2 M nitric acid* and 10 ml of *0.1 M silver nitrate* and titrate with *0.05 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator and shaking vigorously as the end-point is approached. Repeat the operation without the substance under examination; the difference between the titrations represents the number of ml of *0.05 M silver nitrate* required.

1 ml of *0.05 M silver nitrate* is equivalent to 0.003995 g of Br.

For Chlorine

Burn the specified quantity of the substance under examination in the prescribed manner using 20 ml of *1 M sodium hydroxide* as the absorbing liquid. When the process is complete, add 2.5 ml of *nitric acid*, 2.5 ml of *water* and 10 ml of *0.1 M silver nitrate* and titrate with *0.05 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator and shaking vigorously as the end-point is approached. Repeat the operation without the substance under examination; the difference between the titrations represents the number of ml of *0.05 M silver nitrate* required.

1 ml of *0.05 M silver nitrate* is equivalent to 0.001773 g of Cl.

For Fluorine

Burn the specified quantity of the substance under examination in the prescribed manner using 20 ml of *water* as the absorbing liquid. When the process is complete, add sufficient *water* to produce 100.0 ml. To 2.0 ml of this solution add 50 ml of *water*, 10 ml of *alizarine fluorine blue solution*, 3 ml of a solution containing 12 per cent w/v of *sodium acetate* and 6 per cent v/v of *glacial acetic acid*, 10 ml of *cerous nitrate solution* and sufficient *water* to produce 100.0 ml. Allow to stand in the dark for 1 hour and measure the *absorbance* of a 4-cm layer of the resulting solution at about 610 nm (2.4.7), using as the blank a solution prepared in the same manner but using 2.0 ml of *water* in place of the solution and beginning at the words "To 2.0 ml...". Calculate the flourine content from a reference curve prepared by treating suitable aliquots of a solution of *sodium fluoride* in the manner described above, beginning at the words "add 50 ml of *water*".

For Iodine

Burn the specified quantity of the substance under examination in the prescribed manner using a mixture of 10 ml of *water* and 2 ml of *1 M sodium hydroxide* as the absorbing liquid. When the process is complete, add an excess (5 to 10 ml) of *acetic bromine solution* and allow to stand for 2 minutes. Remove the excess of bromine by the addition of *formic acid* (0.5 to 1.0 ml), rinse the sides of the flask with *water* and sweep out any bromine vapour above the liquid with a current of air. Add 1 g of *potassium iodide* and titrate with *0.02 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.02 M sodium thiosulphate* is equivalent to 0.000423 g of I.

For Selenium

Diaminonaphthalene solution. Dissolve 0.1 g of *2,3-diaminonaphthalene* and 0.5 g of *hydroxylamine hydrochloride* in *0.1M hydrochloric acid*, and dilute to 100 ml with the same solvent (*NOTE — Use freshly prepared solution*).

Test solution. Burn the specified quantity of the substance under examination in the prescribed mannerusing 25 ml of *dilute nitric acid* (1 in 30) as the absorbing liquid. When the process is complete, add a few ml of *water* in the cup, loosen the stopper, and rinse the stopper, the specimen holder, and the sides of the flask with *water*. Transfer the solution with the aid of about 20 ml of *water* to a 150-ml beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Reference solution. Dissolve 40 mg of metallic selenium in 100 ml of *dilute nitric acid* (1 in 2) in a 1000-ml volumetric flask, gently heat on a water-bath if necessary, dilute to volume with *water*. Dilute 5.0 ml of the solution to 200.0 ml with *water* (1 ml contains 1 µg of *selenium* (1 ppm)). To 6.0 ml of the solution into a 150-ml beaker, add 25 ml of *dilute nitric acid* (1 in 30) and 25 ml of *water*.

Treat the reference solution, the test solution and the reagent blank consisting 25 ml of *dilute nitric acid* (1 in 30) and 25 ml of *water*, at the same time and in the same manner, using *ammonium hydroxide solution* (1 in 2) adjusted to pH 2.0. Dilute with *water* to 60 ml and transfer to a low-actinic separator with the aid of 10 ml of *water*, adding 10 ml of *water* for rinsing to the separator. Add 0.2 g of *hydroxylamine hydrochloride*, and swirl to dissolve, immediately add 5.0 ml of diaminonaphthalene solution, insert the stopper, and swirl

to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 ml of *cyclohexane*, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed water. Determine the absorbance of the cyclohexane extracts of the test solution and the reference solution in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm (2.4.7), using the cyclohexane extract of the reagent as the blank, and compare the absorbances. The absorbance of the test solution is not more than that of the reference solution.

NOTE — Clear combustion of the test material is an important factor in conducting the test. For compounds that burn poorly and produce soot, the addition of magnesium oxide usually results in more thorough combustion and reduces ash formation. Where the need to add magnesium oxide has been identified, it is specified in the individual monograph.

For Sulphur

Method I *(in the absence of halogens and phosphorus)* — Burn the specified quantity of the substance under examination in the prescribed manner using 10 ml of *water* and 0.1 ml *hydrogen peroxide solution (100 vol)* as the absorbing liquid. When the process is complete, cool the solution in ice for about 15 minutes. Gently boil for 2 minutes, cool and add 50 ml of *ethanolic acetic-ammonia buffer pH 3.7*. Titrate with *0.05 M barium perchlorate* using 0.3 ml of *alizarin red S solution* as indicator, until the solution becomes orange-pink in colour.

1 ml of *0.05 M barium perchlorate* is equivalent to 0.001603 g of S.

Method II *(in the presence of halogens or phosphorus)* — Burn the specified quantity of the substance under examination in the prescribed manner using 15 ml of *water* and 1 ml of *hydrogen peroxide solution (20 vol)* as the absorbing liquid. When the process is complete, boil the solution for 10 minutes, cool and add 60 ml of *ethanol (95 per cent)*. Titrate the solution with *0.01 M barium perchlorate*, using a 0.1 ml of 0.2 per cent w/v solution of *thoron* and 0.1 ml of a 0.0125 per cent w/v solution of *methylene blue* as indicator, until the yellow colour changes to pink.

1 ml of *0.01 M barium perchlorate* is equivalent to 0.0003206 g of S.

If the temperature at which the determinations prescribed under Method I and II are performed differs from that at which the barium perchlorate solution was standardised, the titrant volumes are corrected by applying the expression

$$V_c = V[1 + 0.0008(t_1 - t_2)]$$

where, V_c = the corrected volume of titrant,
V = the volume of titrant used,
t_1 = the temperature of the titrant during standardisation,
t_2 = the temperature of the titrant during the determination.

2.3.35. Peroxide Value

The peroxide value is the number of milliequivalents of active oxygen that expresses the amount of peroxide contained in 1000 g of the substance.

Method

Unless otherwise specified in the individual monograph, weigh accurately about 5 g of the substance under examination, transfer to a 250-ml glass-stoppered conical flask, add 30 ml of a mixture of 3 volumes of *glacial acetic acid* and 2 volumes of *chloroform*, swirl until dissolved and add 0.5 ml of *saturated potassium iodide solution*. Allow to stand for exactly 1 minute, with occasional shaking, add 30 ml of *water* and titrate gradually, with continuous and vigorous shaking, with *0.01 M sodium thiosulphate* until the yellow colour almost disappears. Add 0.5 ml of *starch solution* and continue the titration, shaking vigorously until the blue colour just disappears (a ml). Carry out a blank titration omitting the substance under examination (b ml). The volume of *0.01 M sodium thiosulphate* in the blank determination must not exceed 0.1 ml.

Calculate the peroxide value from the expression

$$\text{Peroxide value} = \frac{10(a-b)}{w}$$

where, w = weight, in g, of the substance.

2.3.36. Phenol in Vaccines and Antisera

Homogenise the preparation under examination. Dilute an appropriate volume with *water* to give a solution containing about 15 µg of phenol per ml. To 5.0 ml of the resulting solution add 5 ml each of *buffer solution pH 9.0*, *4-aminophenazone solution* and *potassium ferricyanide solution*. Allow to stand for 10 minutes and measure the absorbance of the resulting solution at about 546 nm (2.4.7). Take 0.1 ml of the preparation under examination containing about 50 µg per ml to 200 µg per ml of phenol in a test tube. Add sufficient borate buffer pH-9 to produce 3.0 ml of solution. To this solution add 3 ml of freshly prepared *4-aminoantipyrine solution (0.1 per cent)* and 3 ml *potassium ferricyanide solution (1 per cent)*. Measure the absorbance (2.4.7) of the resulting solution at 550 nm using a blank prepared in the same manner using 0.1 ml of distilled water in place of the preparation under examination. Calculate the phenol content from the absorbance obtained using calibration curve prepared curve prepared by repeating the operation using 0.1 ml of series of phenol solution containing 50 µg; 100 µg, 150 µg, 200 µg, 250 µg and 300 µg of phenol per ml respectively.

e absorbance obtained from the test
ce solution at about 525 nm against

mg, of the steroid in the 20-ml aliquot
n the expression

At / As x Cs

sorbance of the test solution, As is the
eference solution and Cs is the quantity,
nce substance in the 20-ml aliquot of the

ntity of the steroid in the substance under
he basis of the aliquot of the test solution
y and from the declared content of the steroid
e reference substance.

saponifiable Matter

fiable matter consists of substances present in
which are not saponifiable by alkali hydroxides
mined by extraction with an organic solvent of a
he saponified substance under examination.

herwise specified in the individual monograph,
about 5 g of the substance under examination,
accurately weighed, into a 250-ml flask fitted with a reflux condenser. Add a solution of 2 g of *potassium hydroxide* in 40 ml of *ethanol (95 per cent)* and heat on a water bath for 1 hour, shaking frequently. Transfer the contents of the flask to a separating funnel with the aid of 100 ml of hot *water* and, while the liquid is still warm, shake very carefully with three quantities, each of 100 ml, of *peroxide-free ether*. Combine the ether extracts in a second separating funnel containing 40 ml of water, swirl gently for a few minutes, allow to separate and reject the lower layer. Wash the extract with two quantities each of 40 ml, of *water* and with three quantities, each of 40 ml, of a 3 per cent w/v solution of *potassium hydroxide*, each treatment being followed by a washing with 40 ml of *water*. Finally, wash the ether layer with successive quantities, each of 40 ml, of *water* until the aqueous layer is not alkaline to *phenolphthalein solution*. Transfer the ether layer to a weighed flask, washing out the separating funnel with *peroxide-free ether*. Distill off the ether and add to the residue 6 ml of *acetone*. Remove the solvent completely from the flask with the aid of a gentle current of air. Dry at 100° to 105° for 30 minutes. Cool in a desiccator and weigh the residue. Calculate the unsaponifiable matter as per cent w/w.

Dissolve the residue in 20 ml of *ethanol (95 per cent)*, previously neutralised to *phenolphthalein solution* and titrate with *0.1 M ethanolic potassium hydroxide*. If the volume of

before titration the ...
by exposing it in a shallow dish ...
24 hours before weighing the sample.

2.3.38. Assay of Steroids

Note—The following procedure is applicable for determination of those Pharmacopoeial steroids that possess reducing functional groups such as α-ketols.

Test solution. Prepare as directed in the individual monograph.

Reference solution. Weigh accurately a suitable quantity of the reference substance specified in the individual monograph, previously dried under the conditions specified in the monograph, and dissolve in a suitable volume of *aldehyde-free ethanol*. Dilute quantitatively and stepwise with *aldehyde-free ethanol* to obtain a solution containing about 10 µg of the steroid per ml.

Method

Into a glass-stoppered, 50-ml conical flask add 20.0 ml of the test solution. Into two similar flasks add 20.0 ml of the Reference solution and 20.0 ml of *aldehyde-free ethanol* (blank), respectively. To each flask add 2.0 ml of *blue tetrazolium solution* and mix; to each flask add 2.0 ml of a mixture of *tetramethylammonium hydroxide solution (10 per cent)* and 90 volumes of *aldehyde-free ethanol*, mix and allow to stand in the dark at a temperature between 25° and 35°. At the end of exactly 90 minutes add to each flask 1.0 ml of *glacial acetic*

0.1 M ethanolic potassium hydroxide exceeds 0.2 ml, the amount weighed cannot be taken as the unsaponifiable matter, and the test must be repeated.

2.3.40. Sulphur Dioxide

Use Method A unless otherwise directed.

Method A

Apparatus

A round-bottomed flask of 1000 to 1500 ml capacity is connected with a water-cooled reflux condenser whose upper end is connected with two absorption tubes in series. The flask is provided with a gas inlet tube which reaches nearly to the bottom of the flask. Each absorption tube contains 10 ml of *hydrogen peroxide solution (20 vol)* neutralised with *0.1 M sodium hydroxide* using *bromophenol blue solution* as indicator.

Procedure

Place in the flask 500 ml of *water* and 20 ml of *hydrochloric acid*. Connect the flask with the condenser and absorption tubes, pass through it a steady current of *nitrogen* or *carbon dioxide* which has been bubbled through *sodium carbonate solution* and gradually heat the liquid until it boils. Maintain the current of *nitrogen* or *carbon dioxide*, allow the solution to boil for about 10 minutes and then cool the flask by gradual immersion in water. Introduce, by momentarily removing the stopper of the flask, 50 to 100 g of the substance under examination, heat gently and boil for 45 minutes. Turn off the current of *nitrogen* or *carbon dioxide*, disconnect the absorption tubes and titrate the contents with *0.1M sodium hydroxide*.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.003203 g of SO_2.

Repeat the operation without the substance under examination; the solution in the absorption tubes remains neutral.

Method B

Apparatus

A 500-ml three-necked round-bottomed flask is fitted with a water-cooled reflux condenser, 200 mm long, the upper end of which is connected to an absorption tube. The flask is fitted with a 100-ml dropping funnel and a gas inlet tube which reaches nearly to the bottom of the flask. The absorption tube contains 10 ml of *hydrogen peroxide solution (10 vol)* previously neutralised to *bromophenol blue solution*.

Procedure

Place 150 ml of *water* in the flask and pass a stream of *carbon dioxide* at a rate of 100 ml per minute for 15 minutes. Connect the absorption tube and without interrupting the flow of *carbon dioxide* introduce through the funnel the prescribed quantity of the substance under examination and 80 ml of *2 M hydrochloric acid*. Boil for 1 hour, disconnect the absorption tube and stop the flow of *carbon dioxide*. Wash the contents of the absorption tube into a 250-ml conical flask, heat on a water-bath for 15 minutes and allow to cool. Titrate with *0.1 M sodium hydroxide* using *bromophenol blue solution* as indicator until the colour changes from yellow to violet-blue.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.003203 g of SO_2.

2.3.41. Assay of Vitamin A

The following methods for the determination of the potency of vitamin A in pharmacopoeial preparations are based on the measurement of the ultra-violet spectrum of the vitamin at the specified wavelength. The potency of vitamin A is expressed in terms of the Unit, which is 0.344 µg of *all-trans*-vitamin A acetate, equivalent to 0.3 µg of *all-trans*-vitamin A alcohol.

A simple measurement of the absorption maximum is not sufficient because in addition to vitamin A, other substances may contribute to the measured absorbance. The irrelevant absorption of such substances may be taken care of under certain conditions (given below) by the use of correcting equations. In other cases, where these conditions are not satisfied, preliminary treatment by chemical or physical methods or by a combination of these methods may be necessary before the following spectrophotometric methods are used to give a valid assay.

The assay should be carried out as speedily as possible and care must be taken to avoid exposure to actinic light and oxidising agents and to maintain to the extent possible an atmosphere of nitrogen above the solutions.

Since the position of the absorption maximum is an important criterion and since the correcting equations require measurements at exact wavelengths, it is essential that the wavelength scale of the spectrophotometer is checked immediately before the assay (2.4.7). The mercury lines at 313.16 nm and 334.15 nm provide suitable points and for convenience the setting of the instrument on these lines may be related to its setting on the hydrogen lines at 379.7 nm and 496.1 nm. The precision of a corrected absorbance is appreciably less than that of the three directly determined absorbances from which it is calculated. The absorbance measurements therefore require special care and not fewer than two assays should be performed.

Method A

This method is suitable for preparations which contain vitamin A as an ester and in a form directly soluble in *cyclohexane*.

Procedure

Dissolve an accurately weighed quantity of the substance under examination in sufficient *cyclohexane* to give a solution containing 9 to 15 Units of vitamin A per ml. Determine the wavelength of maximum absorption. Measure the absorbances (2.4.7) of the solution against the cyclohexane at the wavelength given in Table 1. Calculate the absorbances at the wavelengths specified, as fractions relative to that at 328 nm. Calculate also the absorbance at 328 nm in terms of specific absorbance for the sample.

Table 1

Wavelength (nm)	Relative absorbance
300	0.555
316	0.907
328	1.000
340	0.811
360	0.299

If the wavelength of maximum absorption lies between 326 and 329 nm and the relative absorbances are within 0.02 of those in Table 1, calculate the vitamin A potency of the sample from the expression

A_{328}(1 per cent, 1 cm) × 1900 = Vitamin A potency in Units per g.

If the wavelength of maximum absorption lies between 326 nm and 329 nm but the relative absorbances are not within 0.02 of those in the table, calculate a corrected absorbance at 328 nm by applying the observed values to the equation

$$A_{328}(\text{corr.}) = 3.52(2A_{328} - A_{316} - A_{340})$$

If the corrected absorbance lies within ± 3.0 per cent of the uncorrected absorbance, ignore the corrected absorbance and calculate the potency from the uncorrected absorbance.

If the corrected absorbance lies within −15 per cent and −3 per cent of the uncorrected absorbance, calculate the potency from the corrected absorbance.

If the corrected absorbance lies outside −15 per cent to +3 per cent of the uncorrected absorbance or if the wavelength of maximum absorption does not lie between 326 nm and 329 nm, the substance under examination must be dealt with as described under Method B.

Method B

Special Reagents

All-trans-Vitamin A Acetate

Description. A white to very yellow, free flowing crystals.

Solubility. Very soluble in *ethanol (95 per cent)* in *chloroform*, in *ether*, in *light petroleum*, in fats and in fixed oils; practically insoluble in *water*.

Light absorption. Absorbance of a 0.0003 per cent w/v solution in *2-propanol* at about 325 nm, not less that 0.458, in *cyclohexane* at about 327.5, not less that 0.455 and in *ethanol* at about 326 nm, not less than 0.464 (2.4.7).

Melting range. 57° to 60° (2.4.21).

Relative absorbance. Measure the absorbance (2.4.7), of a 0.0003 per cent w/v solution of *cyclohexane* at about 327.5 nm and at the following wavelengths. The relative absorbances, calculated with reference to the absorbance at about 327.5 nm, are within the limits stated in Table 2.

Table 2

Wavelength (nm)	Relative absorbance
300	0.545 to 0.565
312.5	0.845 to 0.865
337.5	0.845 to 0.865
345	0.685 to 0.705
360	0.290 to 0.310

Procedure

Weigh accurately a quantity of the substance under examination containing not less than 500 Units of vitamin A and not more than 1 g of fat. Add about 50 mg of *hydroquinone*, 30 ml of *ethanol* and 3 ml of a 50 per cent w/v solution of *potassium hydroxide*. Boil gently under a reflux condenser in a stream of *oxygen-free nitrogen* for 30 minutes, cool rapidly and add 30 ml of *water*. Transfer to a separator with the aid of three quantities, each of 50 ml, of *ether* and extract the vitamin A by shaking for 1 minute. After complete separation discard the aqueous layer and wash the extract with four quantities, each of 50 ml, of *water*, mixing very cautiously during the first two washes to avoid the formation of emulsions. Evaporate the separated extract to about 5 ml and remove the remaining solvent in a stream of *oxygen-free nitrogen* without the application of heat. Dissolve the residue in sufficient *2-propanol* to produce a solution containing 9 to 15 Units of vitamin A per ml and measure the absorbance at about 300, 310, 325 and 334 nm. Determine the wavelength of maximum absorption.

If the wavelength of maximum absorption lies between 323 nm and 327 nm and the absorbance at about 300 nm relative to that at about 325 nm does not exceed 0.73, a corrected absorbance is derived from the equation

$$A_{325}(\text{corr.}) = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

Calculate the potency of the sample from the expression

$$A_{325}(1\ \text{per cent, 1 cm}) \times 1830 = \text{Vitamin A potency in Units per g.}$$

If the corrected absorbance lies within ± 3.0 per cent of the uncorrected absorbance, ignore the corrected absorbance and calculate the potency from the uncorrected absorbance.

If the wavelength of maximum absorption lies outside the range 323 nm to 327 nm, or if the relative absorbance at about 300 nm exceeds 0.73, the unsaponifiable fraction of the sample must be further purified by chromatography.

2.3.42. Assay of Vitamin D

The assay should be completed promptly and care should be taken throughout the procedure to keep to a minimum the exposure to air and to actinic light by the use of inert gas and low actinic glassware. All the operations should be carried out in subdued light.

Special Reagents

Adsorbent: A chromatographic grade of kaolin such as Florex XXS or of fuller's earth having a water content corresponding to not less than 8.5 per cent and not more than 9.0 per cent of loss on drying at 105° for 6 hours.

NOTE — Adjust the water content, if necessary, by drying in vaccum at room temperature, restoring the water required and equilibrating by shaking for 2 hours.

Standard preparation of vitamin D. Dissolve 0.01 g of *ergocalciferol RS* or *cholecalciferol RS* (for assaying substances labelled to contain Vitamin D as ergocalciferol or as cholecalciferol respectively) in sufficient *purified 1,2-dichloroethane* to produce 100.0 ml. Dilute 10.0 ml of this solution to 100.0 ml with *purified 1,2-dichloroethane* to give a solution containing 10 µg (400 Units) of vitamin D per ml.

Apparatus

Chromatographic Tubes

Column No. 1. A chromatographic tube (25 cm x 2.5 mm) fitted at the lower end with a sintered glass disc or a small plug of glass wool and a tap.

Column No. 2. A chromatographic tube (20 cm x 5 mm) fitted at the lower end with a sintered glass disc and a tap.

Chromatographic columns

Column No. 1. Shake 200 ml of *2,2,4-trimethylpentane* with sufficient *polyethylene glycol 600* so that, on separation, two layers are obtained. To 100 ml of the upper layer add 25 g of *chromatographic siliceous earth*, shake vigorously to form a thin slurry, add, in small portions and with vigorous stirring, 10 ml of *polyethylene glycol 600* and continue to stir for a further 2 minutes to produce a uniform suspension. Transfer the suspension in small portions to the chromatographic tube, apply gentle suction and pack each portion carefully with the aid of a glass plunger. Add sufficient of the suspension to produce a column 15 cm in length and discard any eluate.

Standardisation of column — Determine the volume in which *ergocalciferol* is recovered from the column by the following method.

Transfer 2 ml of a 0.03 per cent w/v solution of *ergocalciferol* in *2,2,4-trimethylpentane* to the top of the column and rinse it into the column with not more than 5 ml of *trimethylpentane*. Elute the column with *2,2,4-trimethylpentane*, adjusting the rate of flow from the bottom of the column to 2 to 3 ml per minute and collect successive 5 ml fractions of the eluate. Measure the absorbance of each fraction at the maximum at about 263 nm and there from determine the position, relative to volume of eluate, at which the elution of *ergocalciferol* begins and finishes.

Column No. 2. Mix 5 g of *adsorbent* with sufficient *2,2,4-trimethylpentane* to form a slurry and transfer the slurry to the chromatograph tube. Pack the tube carefully with the aid of glass plunger and discard the eluate.

Procedure

Weigh accurately a quantity of the substance under examination equivalent to about 400 Units of vitamin D. For capsules, the mixed contents of 20 capsules may be used as the sample. Add 10 ml of a freshly prepared 0.01 per cent w/v solution of *butylated hydroxytoluene* in *ethanol (95 per cent)*, 15 ml of a 50 per cent w/v solution of *potassium hydroxide* and 5 ml of *ethanol (95 per cent)*. Reflux on a water-bath for 30 minutes, cool, transfer the solution to a separator with the aid of 50 ml of *water*, add 75 ml *ether* and shake vigorously. Allow to separate, transfer the aqueous layer to a second separator and extract with three successive quantities, each of 30 ml, of *ether*, adding each ethereal extract to the liquid in the first separator and finally discarding the aqueous solution.

Pour two successive quantities, each of 100 ml, of *water* through the ethereal solution without shaking and discard the aqueous layers. Add successive quantities, each of 10 ml, of *water* to the ethereal solution, agitate gently each time and discard the aqueous extracts. Continue the process until the aqueous extracts are neutral to *phenolphthalein solution*.

Dry the ethereal solution by stirring with *anhydrous sodium sulphate*, decant the ethereal solution, wash the residue with successive small portions of *ether* and evaporate the combined solution and washings on a water-bath to a volume of about 5 ml. Cool and evaporate to dryness in a current of *nitrogen*. Dissolve the residue in 5.0 ml of *trimethylpentane* to obtain the sample preparation.

Transfer the sample preparation to the top of chromatographic column No. 1 with the aid of 5 ml of *2,2,4-trimethylpentane*; elute the column with *2,2,4-trimethylpentane*, adjusting the rate of flow of eluate from the bottom of the column to 2 to 3 ml per minute, and collect the fraction of eluate estimated to contain the calciferol, as indicated by the standardisation of the column.

Transfer the eluate collected from chromatographic column No. 1 to the top of chromatographic column No. 2, allow the liquid to flow, add to the top of the column 10 ml of *trimethylpentane* and discard the eluate. Elute the column with 50 ml of *benzene*, evaporate the eluate on a water-bath to a volume of about 5 ml, cool, evaporate to dryness in a current of *nitrogen* and dissolve the residue in 4.0 ml of *purified 1,2-dichloroethane* to obtain the sample solution (solution A).

Add 1.0 ml of solution A to each of three tubes. To the first tube add 1.0 ml of a mixture of equal volumes of *purified 1,2-dichloroethane* and *acetic anhydride* and 10.0 ml of *antimony trichloride solution* in *purified 1,2-dichloroethane*. To the second tube add 1.0 ml of *purified 1,2-dichloroethane* and 10.0 ml of *antimony trichloride solution* in *purified 1,2-dichloroethane*. To the third tube add 1.0 ml of the *standard preparation of vitamin D* and 10.0 ml of *antimony trichloride solution* in *purified 1,2-dichloroethane*.

Measure the absorbance of each solution exactly 1 minute after the addition of the *antimony trichloride solution* at the maximum at about 500 nm using *purified 1,2-dichloroethane* as the blank (2.4.7). The amount of vitamin D, in mg, in the weight of the sample taken is given by the expression

$$\frac{0.04\,(A_1 - A_2)}{(A_3 - A_2)}$$

where, A_1 = the absorbance due to the solution in the first tube;

A_2 = the absorbance due to the solution in the second tube;

A_3 = the absorbance due to the solution in the third tube.

1 mg of ergocalciferol or cholecalciferol is equivalent to 40,000 Units of vitamin D.

2.3.43. Water

Method 1. Titrimetric Method

Apparatus

A titration vessel of about 60 ml capacity fitted with two platinum electrodes, about 0.05 sq. cm in area and about 2.5 cm apart a nitrogen inlet tube, a stopper which accommodates the burette tip and a vent tube protected by a suitable desiccant such as *phosphorous pentoxide* or *silica gel*. The substance under examination is introduced through an inlet or side arm that can be closed by a ground stopper. Stirring is done magnetically or by means of a stream of dried *nitrogen* passed through the solution during the titration. The air in the entire system should be kept dry during the titration.

The end-point is determined by amperometry. The circuit consists of a potentiometer of about 2000 ohms connected across a 1.5 V battery. The resistance is adjusted so that an initial low current passes through the electrodes. On adding the reagent the needle of the microammeter shows a deflection but returns immediately to its starting position. At the end point of the titration a slight excess of the reagent produces a deflection that persists for not less than half a minute.

Karl Fischer (KF) reagent. KF reagent is an iodosulphurous compound containing basic substances like pyridine, piperidine etc and may contain imidazole. The reagent and solutions used for preparing the KF should be kept anhydrous and care should be taken throughout the determination to prevent exposure to atmospheric moisture. The reagent should be protected from light and stored in a bottle to which is fitted an automatic burette.

The composition of commercially available Karl Fischer reagents often differs from that above by the replacement of pyridine with other basic compounds. The use of these reagents must be validated in order to verify in each individual case the stoichiometry and the absence of incompatibility between the substance under test and the reagent.

Primary standardisation of the reagent. Place about sufficient volume of *dehydrated methanol* in the titration vessel and add sufficient *KF reagent* to give the characteristic end-point. Add quickly 150 to 350 mg of *disodium tartrate*, $C_4H_4O_6Na_2, 2H_2O$ or suitable amount of *water* in appropriate form, accurately weighed by difference and titrate to the end-point. The water equivalence factor, F, in mg of water per ml of the reagent is given by the expression 0.1566 w/v, where w is the weight, in mg of the disodium tartrate or water and v is the volume, in ml, of the reagent required.

Secondary standardisation of the reagent. The *KF reagent* may alternatively be standardised for each day's use against a water-methanol solution standardised as follows. Add 2.0 ml of *water* to 1000.0 ml of *dehydrated methanol*. Retain a portion of the methanol used for a blank determination. Place 25 ml, accurately measured of the water-methanol solution in the titration vessel and titrate with *KF reagent*. Perform a blank titration on 25 ml accurately measured, of the methanol used and make any necessary correction. The water content in mg per ml of the water-methanol solution is given by the expression $VF/25$ in which V is the volume, in ml, of *KF reagent*

required and *F* is the water equivalent factor of the reagent determined against *sodium tartrate* as directed under Primary standardisation of the reagent.

Follow Method A unless otherwise directed.

Method A. Unless otherwise directed, add about sufficient volume of *dehydrated methanol* to the titration vessel and titrate to the electrometric end point with the *KF reagent*. Transfer quickly the prescribed amount of the substance under examination, accurately weighed, to the titration vessel. Stir for 1 minute and titrate again to the electrometric end point using the *KF reagent*.

The water content of the sample, in mg is given by the expression *SXF*, in which *S* is the volume, in ml of the *KF reagent* used to titrate the sample and *F* is the water equivalent factor.

Method B. This method should be followed for samples that react with difficulty or too slowly for convenient direct titration with the *KF reagent*.

Unless otherwise directed add about sufficient volume of *dehydrated methanol* to the titration vessel and titrate to the electrometric end-point with the *KF reagent*. Transfer quickly the prescribed amount accurately weighed, of the substance under examination to the titration vessel followed by an accurately measured amount of *KF reagent* sufficient to given an excess of about 1 ml. Allow to stand, protected from light, for 1 minute, stirring well. Titrate the excess of the reagent to the electrometric end-point with *dehydrated methanol* to which has been added an accurately known amount of *water* equivalent to about 0.25 per cent w/v.

Calculate the content of water from the expression S x F, where S is the volume, in ml, of the KF reagent used to titrate the sample and F is the equivalence factor.

Unless otherwise directed, express the result as a percentage w/w.

Method 2. Azeotropic Distillation Method

Apparatus

The apparatus (see figure 2.3.43-1) consists of a round-bottomed, 500-ml flask (A) connected by means of a trap (B), 23.5 to 24.0 cm long, to vertical reflux condenser of the straight tube type (C) by ground glass joints. The condenser is approx. 40 cm long and has a bore diameter of not less than 8 mm. The connecting tube (D) is 9 to 11 mm in internal diameter. The receiving tube (E) has a 5 ml capacity and its cylindrical part, 14.6 to 15.6 cm in length, is graduated in 0.1 ml sub divisions. The flask is heated in an oil bath or in an electric mantle. The upper portion of the flask and the connecting tube may be insulated.

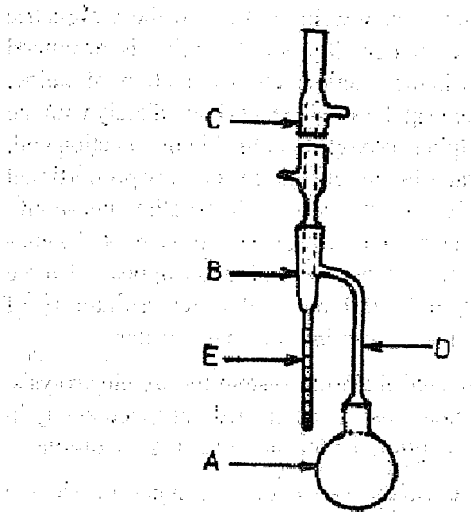


Fig. 2.3.43-1: Apparatus for Determination of water by Azeotropic Distillation.

Before use, the condenser and receiving tube should be cleaned with *chromic acid mixture*, thoroughly rinsed with *water* and dried in an oven.

Method. Weigh accurately a quantity of the substance under examination, that is expected to yield 2 to 4 ml of *water* and transfer to the dry flask. If the substance is semi-solid, weigh it in a metal foil, fold the foil carefully and pass it through the neck of the flask. To prevent bumping, add enough washed and dried sand to cover the bottom of the flask or few capillary melting point tubes, 10 cm long, sealed at the upper end. Add about 200 ml of *prepared toluene*, connect the apparatus and fill the receiving tube (E) with *prepared toluene* poured through the top of the condenser. Heat the flask gently for 15 minutes and when the toluene begins to boil, distil at the rate of about 2 drops per second until most of the water has distilled over. Then increase the rate to about 4 drops per second. When the water has apparently completely distilled over, rinse the inside of the condenser tube with *prepared toluene* with the aid of a tube brush attached to a copper wire and saturated with *prepared toluene*. Continue distillation for 5 minutes, remove the heat and allow the receiving tube to cool to room temperature. If any droplets of water stick to the wall of the receiving tube, scrub them using a copper wire with a rubber band wrapped round it and washed with *prepared toluene*. After complete separation of the water and toluene in the tube, read off the volume of water in the tube and calculate the content of water as a percentage w/w, assuming the weight per ml of water to be 1.0.

Method 3. Coulometric Titration

Principle. The coulometric titration of water is based upon the quantitative reaction of water with sulphur dioxide and iodine in an anhydrous medium in the presence of a base with

sufficient buffering capacity. In contrast to the volumetric method described under Method 1, iodine is produced electrochemically in the reaction cell by oxidation of iodide. The iodine produced at the anode reacts immediately with the water and the sulphur dioxide contained in the reaction cell. The amount of water in the substance is directly proportional to the quantity of electricity up until the titration end-point. When all of the water in the cell has been consumed, the end-point is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, a quantity of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the system by pre-electrolysis. Individual determinations can be carried out successively in the same reagent solution, under the following conditions.

a. each component of the test mixture is compatible with the other components,
b. no other reactions take place,
c. the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is restricted to the quantitative determination of small amounts of water, a range of 10 µg up to 10 mg of water is recommended.

Accuracy and precision of the method are predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

Apparatus. The apparatus consists of a reaction cell, electrodes and magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in a tube (oven) and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

Method. Fill the compartments of the reaction cell with *electrolyte reagent for the determination of water* according to the manufacturer's instructions and perform the coulometric titration to a stable end-point. Introduce the prescribed amount of the substance under examination into the reaction cell, stir for 30 seconds, if not otherwise indicated in the monograph, and titrate again to a stable end-point. In case an oven is used, the prescribed sample amount is introduced into the tube and heated. After evaporation of the water from the sample into the titration cell, the titration is started. Read the value from the instrument's output and calculate if necessary the percentage or amount of water that is present in the substance. When appropriate to the type of sample and the sample preparation, perform a blank titration.

Verification of the accuracy. Between two successive sample titrations, introduce an accurately weighed amount of water in the same order of magnitude as the amount of water in the sample, either as *water* or in the form of *standard solution for the determination of water*, and carryout the coulometric titration. The recovery rate is within the range from 97.5 per cent to 102.5 per cent for an addition of 1,000 µg of H_2O and in the range from 90.0 per cent to 110.0 per cent for the addition of 100 µg of H_2O.

2.3.44. Zinc

NOTE — All reagents used in this test should have as low a content of heavy metals as practicable. All glassware should be rinsed with warm dilute nitric acid followed by water previously distilled in hard or borosilicate glass apparatus. Separators should not be greased with materials that dissolve in chloroform.

Method

Pipette 1 to 5 ml of the preparation under examination into a centrifuge tube graduated at 40 ml. If necessary, add *0.25 M hydrochloric acid* dropwise, to obtain a clear solution. Add 5 ml of *trichloroacetic acid solution* and sufficient *water* to produce 40.0 ml. Mix well and centrifuge.

Pipette into a hard-glass separator a volume of the supernatant liquid equivalent to about 5 to 20 µg of zinc and add *water* to produce 20 ml. Add 1.5 ml of *alkaline ammonium citrate solution* and 35 ml of *dithizone standard solution*. Shake vigorously several times and allow the chloroform to separate. Collect the chloroform extract through a cotton plug placed in the stem of the separator and discard the first few ml. Measure the absorbance of the extract at about 530 nm (2.4.7), using as the blank an extract obtained by repeating the determination omitting the preparation under examination.

Calculate the amount of zinc by reference to standard graph obtained by using 0.5 ml, 1.0 ml, 1.5 ml and 2.0 ml of *zinc standard solution (10 ppm Zn)* and repeating the determination as outlined above.

2.3.45. Ethanol

The ethanol content of a liquid is expressed as the number of volumes of ethanol contained in 100 volumes of the liquid, the volumes being measured at 24.9° to 25.1°. This is known as the

"percentage of ethanol by volume". The content may also be expressed in grams of ethanol per 100 g of the liquid. This is known as the "percentage of ethanol by weight".

Use Method I or Method II, as appropriate, unless otherwise specified in the individual monograph.

Method I

Determine by gas chromatography (2.4.13).

Test solution (a). Dilute a volume of the preparation under examination with *water* to contain between 4.0 and 6.0 per cent v/v of *ethanol*.

Test solution (b). Prepare in the same manner as test solution (a) but adding sufficient of *1-propanol* (internal standard) to produce a final concentration of 5.0 per cent v/v.

Reference solution. A 5.0 per cent v/v of *ethanol* and 5.0 per cent v/v of *1-propanol* (internal standard).

Chromatographic system

- a glass column 1.5 m x 4 mm, packed with porous polymer beads (100 to 120 mesh),
- temperature:
  column 150°,
  inlet port and detector 170°,
- flow rate: 30 ml per minute, using nitrogen as carrier gas.

Calculate the percentage content of ethanol from the ratio of the area of the peaks due to ethanol and internal standard in the chromatogram obtained with test solution (b) and reference solution.

Method II

For preparations where the use of Industrial Methylated Spirit is permitted in the monograph, determine the content of ethanol as described in Method I but using as following solution

Test solution (a). A volume of the preparation under examination diluted with *water* to contain between 4.0 and 6.0 per cent v/v of total ethanol and methanol.

Determine the concentration of methanol in the following manner. Using the chromatographic condition as described under Method I but using the following solutions.

Test solution (a). Dilute a volume of the preparation under examination with *water* to contain between 0.2 per cent and 0.3 per cent v/v of *methanol*.

Test solution(b). Prepare in the same manner as test solution (a) but adding sufficient of the *1-propanol* (internal standard) to produce a final concentration of 0.25 per cent v/v.

Reference solution. A 0.25 per cent v/v of *methanol* and 0.25 per cent v/v of *1-propanol* (internal standard).

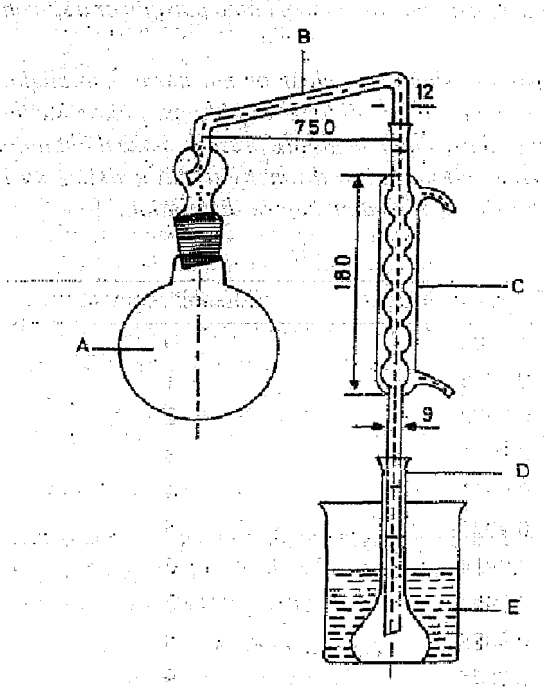
The sum of the contents of ethanol and methanol is within the range specified in the individual monograph and the ratio of the content of methanol to that of ethanol is commensurate with Industrial Methylated Spirit having been used.

Calculate the percentage content of ethanol from the ratio of the area of the peaks due to ethanol and internal standard in the chromatogram obtained with test solution (b) and reference solution.

Method III

This method is intended only for certain liquid preparations containing ethanol. Where the preparation contains dissolved substances that may distil along with ethanol Method IIIB or IIIC must be followed.

Apparatus



(Dimensions in mm)

Fig. 2.3.45-1: Apparatus for Determination of Ethanol by Distillation method

The apparatus (see Fig.2.3.45-1) consists of a round-bottomed flask (A) fitted with a distillation head (B) with a steam trap and attached to a vertical condenser (C). A tube is fitted to the lower part of the condenser and carries the distillate into the lower part of a 100-ml or 250-ml volumetric flask (D). The volumetric flask is immersed in a beaker (E) containing a mixture of ice and water during the distillation. A disc with a circular aperture 6 cm in diameter is placed under the distillation flask (A) to reduce the risk of charring of any dissolved substances.

Method IIIA

Transfer 25 ml of the preparation under examination, accurately measured at 24.9° to 25.1°, to the distillation flask. Dilute with 150 ml of *water* and add a little *pumice powder*. Attach the distillation head and condenser. Distil and collect not less than 90 ml of the distillate into a 100-ml volumetric flask. Adjust

the temperature to 24.9° to 25.1° and dilute to volume with *distilled water* at 24.9° to 25.1°. Determine the relative density at 24.9° to 25.1° (2.4.29). The values indicated in column 2 of the table (see below) are multiplied by 4 in order to obtain the percentage of ethanol by volume contained in the preparation. If the specific gravity is found to be between two values the percentage of ethanol should be obtained by interpolation. After calculation of the ethanol content, report the result to one decimal place.

NOTES — (1) If excessive frothing is encountered during distillation, render the solution strongly acid with phosphoric acid or treat with a small amount of liquid paraffin or a silicone oil.

(2) The distillate should be clear or not more than slightly cloudy. If it is turbid or contains oily drops, follow Method IIIC. When steam-volatile acids are present, make the solution just alkaline with 1 M sodium hydroxide using solid phenolphthalein as indicator before distillation.

Table

Specific gravity at 25°	Ethanol* content
1.0000	0
0.9985	1
0.9970	2
0.9956	3
0.9941	4
0.9927	5
0.9914	6
0.9901	7
0.9888	8
0.9875	9
0.9862	10
0.9850	11
0.9838	12
0.9826	13
0.9814	14
0.9802	15
0.9790	16
0.9778	17
0.9767	18
0.9756	19
0.9744	20
0.9733	21
0.9721	22
0.9710	23
0.9698	24
0.9685	25

* Percent v/v at 15.56°.

Method IIIB

Follow this method or the following one if the preparation under examination contains appreciable proportions of volatile materials other than ethanol and water.

Mix 25 ml of the preparation, accurately measured at 24.9° to 25.1°, with about 100 ml of *water* in a separating funnel. Saturate this mixture with *sodium chloride*, add about 100 ml of *hexane* and shake vigorously for 2 to 3 minutes. Allow the mixture to stand for 15 to 20 minutes. Run the lower layer into the distillation flask, wash the hexane layer in the separating funnel by shaking vigorously with about 25 ml of *sodium chloride solution*, allow to separate and run the wash liquor into the first saline solution. Make the mixed solutions just alkaline with *1 M sodium hydroxide* using solid *phenolphthalein* as indicator, add a little *pumice powder* and 100 ml of *water*, distil 90 ml and determine the percentage v/v of ethanol by Method III A beginning at the words "Adjust the temperature...".

Method IIIC

Transfer 25 ml of the preparation, accurately measured at 24.9° to 25.1°, to the distillation flask. Dilute with 150 ml of *water* and add a little *pumice powder*. Attach the distillation head and condenser. Distil and collect about 100 ml. Transfer to a separating funnel and determine the percentage v/v of ethanol by Method IIIB beginning at the words "Saturate this mixture...".

2.3.46. Assay of Insulins

Use Method A or Method B, as appropriate.

Method A

Determine by liquid chromatography (2.4.14).

Test solution. Prepare as directed in the individual monograph.

Reference solution (a). For the assay of insulin preparations containing 100 Units per ml.

For a preparation containing a single species of insulin, dissolve in *0.01 M hydrochloric acid*, as appropriate, a defined quantity of *human insulin RS* or *porcine insulin RS*, or of *bovine insulin RS*, to obtain a concentration of 4.0 mg per ml. For a preparation containing both bovine and porcine insulins, mix 1.0 ml of a solution containing 4.0 mg of *bovine insulin RS* per ml of *0.01 M hydrochloric acid* and 1.0 ml of a solution containing 4.0 mg of *porcine insulin RS* per ml of *0.01 M hydrochloric acid*.

Reference solution (b). For the assay of insulin preparations containing 40 Units per ml.

Dilute 4.0 ml of reference solution (a) to 10.0 ml with *0.01 M hydrochloric acid*.

Reference solution (c). Dissolve the contents of a vial of *human insulin RS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg per ml.

Reference solution (d). Dissolve the contents of a vial of *porcine insulin RS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg per ml.

Reference solution (e). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *0.01 M hydrochloric acid.*

Reference solution (f). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *0.01 M hydrochloric acid.*

Resolution solution. Mix 1.0 ml of reference solution (c) and 1.0 ml of reference solution (d).

Maintain the solutions at 2° to 10° and use within 48 hours. If an automatic injector is used, maintain the temperature at 2° to 10°.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase A. dissolve 28.4 g of *anhydrous sodium sulphate* in *water* and dilute to 1000 ml with the same solvent; add 2.7 ml of *phosphoric acid*; adjust the pH to 2.3, if necessary with *ethanolamine*; filter and degas,

  B. mix 550 ml of mobile phase (a) with 450 ml of *acetonitrile*; warm the solution to a temperature not lower than 20° in order to avoid precipitation (mixing of mobile phase (a) with acetonitrile is endothermic; filter and degas,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Elute with a mixture of 42 volumes of mobile phase (a) and 58 volumes of mobile phase (b), adjusted if necessary.

Inject the resolution solution and reference solution (d). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (d) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.

Inject the test solution and 20 µl of either reference solutions (a) and (e), for insulin preparations containing 100 Units per ml, or 20 µl of reference solutions (b) and (f), for insulin preparations containing 40 Units per ml. If necessary, make further adjustments of the mobile phase in order to ensure that the antimicrobial preservatives present in the test solution are well separated from the insulin and show shorter retention times. A small reduction in the concentration of acetonitrile increases the retention time of the insulin peaks relatively more than those of the preservatives. If necessary, after having carried out the chromatography of a solution wash the column with a mixture of equal volumes of *acetonitrile* and *water* for a sufficient time to ensure elution of any interfering substances before injecting the next solution. The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (a) or (b) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) or (f). If this test fails, adjust the injection volume between 10 µl and 20 µl, in order to be in the linearity range of the detector.

Calculate the content of insulin plus A21 desamido insulin from the area of the peak due to the bovine, porcine or human insulin and that of any peak due to the A21 desamido insulin, using the declared content of insulin plus A21 desamido insulin in *bovine insulin RS*, *porcine insulin RS* or *human insulin RS*, as appropriate. For preparations containing both bovine and porcine insulin use the sum of the areas of both the bovine and porcine insulin peaks and of the peaks due to the A21 desamido insulin[1] derivatives.

[1]100 Units are equivalent to 3.47 mg of human insulin, to 3.45 mg of porcine insulin and to 3.42 mg of bovine insulin.

Method B

Determination by liquid chromatography (2.4.14) as described under Assay of Insulins (2.3.46) Method A, following the conditions as described below:

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0 - 30	42	58
30 - 44	42 - 11	58 - 89
44 - 50	11	89
50 - 55	42	58

2.3.47. Peptide Mapping

Peptide mapping is an identity test for proteins, especially those obtained by recombinant DNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner. It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of

the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. 4 principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 1 – Examples of cleavage agents

Type	Agent	Specificity
Enzymatic	Trypsin	C- terminal side of Arg and Lys
	Chymotrypsin	C-terminal side of hydrophobic residues (e.g. Leu, Met, Ala, aromatics)
	Pepsin	Non-specific digest
	Lysyl endopeptidase (Lys-C endopeptidase)	C-terminal side of Lys
	Glutamyl endopeptidase (from *S. aureus* strain V8)	C-terminal side of Glu and Asp
	Peptidyl-Asp metallo-endoproteinase Asp-N)	N-terminal side of Asp
	Clostripain	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thio-cyanobenzoic acid	N-terminal side of Cys
	O-Idosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

Pretreatment of sample. Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Dalton, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the cleavage agent. Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the protein. Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from excipients and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultra filtration, column chromatography and lyophilization. Other

pretreatments, such as the addition of chaotropic agents (e.g. urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the peptide map due to side reactions occurring during the digestion reaction, such as non-specific cleavage, deamidation, disulfide isomerisation, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N-terminal* side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g. at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of optimal digestion conditions. Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

(a) **pH.** The pH of the digestion mixture is empirically determined to ensure the optimization of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction must not alter the chemical integrity of the protein during the digestion and must not change during the course of the fragmentation reaction.

(b) **Temperature.** A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4°, because at higher temperatures it will precipitate during digestion.

(c) **Time.** If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 hours to 30 hours. The reaction is stopped by the addition of an acid which does not interfere in the map or by freezing.

(d) **Amount of cleavage agent used.** Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e. 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent is added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping, the separation step. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2. In this section, a most widely used reversed phase HPLC method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC grade solvents and water that are commercially available are recommended for reversed phase HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre and post pump filtration is also recommended.

Table 2. Techniques used for the separation of the peptides

Reversed phase high performance liquid chromatography (HPLC)
Ion-exchange chromatography (IEC)
Hydrophobic interaction chromatography (HIC)
Polyacrylamide gel electrophoresis (PAGE), non-reducing
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
Capillary electrophoresis (CE)
Paper chromatography high voltage (PCHV)
High voltage paper electrophoresis (HVPE)

Chromatographic column. The selection of a chromatographic column is empirically determined for each protein. Columns with 10 nm or 30 nm pore size with silica support can give optimal separation.

Isocratic elution. Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention

times of peaks in peptide maps must not be used in isocratic HPLC systems.

Other parameters. Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 - 2.0 ml per minute, and the detection of peptides is performed with a UV detector at 200 - 230 nm. Other methods of detection have been used (e.g. post column derivatisation), but they are not as robust or versatile as UV detection.

Validation. This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint has been achieved is shown by comparison with a reference standard, which is treated in the same manner as the test protein. The use of a reference substance in parallel with the test protein is critical in the development and establishment of system suitability limits. In addition, a chromatogram is included with the reference substance for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference substance for the specified protein. The use of a digested reference substance for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a reference substance can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters, such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency, may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference substance for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are to be expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retentions, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and reference substance digest. If all peaks in the sample digest and in the reference substance digest have the same relative retentions and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retentions are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and reference substance digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches are, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference substance and sample digest fragments.

The numerical comparison of the peak retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using 1 peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference substance. The possibility of auto-

hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterisation of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Elutes corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase and matrix-assisted laser desorption ionization coupled to time- of-flight analyzer (MALDI-TOF) can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95 per cent of the theoretical composition of the protein structure.

2.3.48. Thiomersal

Method A

Take 0.1ml of the preparation under examination containing about 50 µg per ml of *thiomersal* in a test-tube add sufficient *distilled water* to produce 1.0 ml. To this solution add 1.0 ml of *acetone*, 1.0 ml of a freshly prepared 0.001 per cent w/v solution of *dithizone in acetone* and 0.1ml of *sodium hydroxide* (*50 per cent w/v*). Measure the absorbance (2.4.7) of the resulting solution at 538 nm using a blank prepared in the same manner using 0.1ml of *distilled water* in place of the preparation under examination. Calculate the thiomersal content from the absorbance obtained, using calibration curve prepared by repeating the operation using 0.1 ml of a series of *thiomersal* solutions containing 25 µg, 50 µg, 75 µg, 100 µg and 125 µg per ml.

Method B

To an accurately measured volume of the preparation being examined containing the equivalent of 50 µg of Thiomersal taken in a separating funnel add sufficient of a 1 per cent w/v solution of *ammonium acetate*, adjusted to pH 6.0 to produce 10 ml. Add 10 ml of freshly prepared 0.001 per cent w/v solution of *dithizone* in *chloroform* and shake vigorously for 45 second. Separate the chloroform layer, filter through absorbent cotton and measure the absorbance of the filtrate at the maximum at about 480 nm using as the blank a solution prepared by treating the same volume of *water* as of the preparation being examined in the same manner beginning at the words "add sufficient of a 1 per cent solution of ammonium acetate ……". Calculate the content of $C_9H_9HgNaO_2S$ from the absorbance obtained by repeating the operation using an aqueous solution containing the equivalent of 50 µg of *Thiomersal* RS.

Method C

Any other validated method approved by National Control Laboratory.

2.3.49. Protein

Use any of the following methods.

Method A

Biuret method

Dilute an appropriate volume with *distilled water* to give a solution containing about 5 mg of protein per ml. To 1.0 ml of resulting solution in a test tube, add 4 ml of *biuret reagent*

(prepared by dissolving 6 g of *sodium potassium tartrate* and 1.5 g of *cupric sulphate* in 500 ml of *distilled water*. Add with constant stirring 300 ml of 10 per cent *sodium hydroxide* and make the final volume to 1000 ml by adding 5 g of *potassium iodide*. Measure the absorbance (2.4.7) of the resulting test solution at 550 nm using blank prepared in the same manner by taking 1.0 ml of *distilled water*. Calculate the *protein* content from the absorbance obtained, using calibration curve prepared by repeating the operation using 1.0 ml of each of a series of bovine serum albumin solution containing 1.0 mg, 2.0 mg, 3.0 mg, 4.0 mg and 5.0 mg per ml of *protein* respectively.

Method B

Lowry's Method

Prepare a stock solution of the standard protein (example, bovine serum albumin fraction V) containing 2 mg per ml protein in *distilled water*. Store the stock solution frozen at -20°. Prepare standards by diluting the stock solution with *distilled water* to give the protein concentrations of 0 µg, 10.0 µg, 20.0 µg, 50.0 µg, 100.0 µg, 200.0 µg, 500.0 µg, 1000.0 µg and 2000.0 µg per ml.

Prepare the complex forming reagent immediately before use by mixing the following stock solutions in the proportion of 100:1:1 (by volume) respectively.

Solution (a). 2 per cent w/v solution of *sodium carbonate* in *distilled water*.

Solution (b). 1 per cent w/v solution of *copper sulphate* in *distilled water*.

Solution (c). 2 per cent w/v solution of *sodium tartrate* in *distilled water*.

Take 0.1 ml of test sample or standard protein and add 0.1 ml of *2 M sodium hydroxide* and hydrolyze the mixture at 100° for 10 minutes in a heating block or boiling water bath. Cool the mixture to room temperature and add 1 ml of freshly mixed complex forming reagent. Allow the solution to stand at room temperature for 10 minutes. Add 0.1 ml of *Folin's reagent*, mix well and let the mixture stand at room temperature for 30 to 60 minutes. Read the absorbance at 750 nm (2.4.7). Determine the protein concentration of the test sample by plotting a standard curve of absorbance as a function of concentration of the standard protein.

2.3.50. Fatty Acid Composition by Gas Chromatography

The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil under examination by gas chromatography (2.4.13).

Method A. *This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.*

Test solution. When prescribed in the monograph, dry the oil under examination before the methylation step. Weigh 1.0 g of the oil into a 25-ml round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 ml of *anhydrous methanol* and 0.2 ml of a 6.0 per cent w/v solution of *potassium hydroxide* in *methanol*. Attach the reflux condenser, pass *nitrogen* through the mixture at a rate of about 50 ml per minute, shake and heat to boiling for about 10 minutes. When the solution is clear, continue heating for a further 5 minutes. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 ml of *heptane* and transfer the rinsings to the separating funnel and shake. Add 10 ml of a 20 per cent w/v solution of *sodium chloride* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulphate*. Allow to stand, then filter.

Reference solution (a). Prepare 0.5 g of the mixture of calibrating substances with the composition described in one of the tables, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 1). Dissolve in *heptane* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *heptane*.

Reference solution (c). Prepare 0.5 g of a mixture of fatty acid methyl esters that corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance under examination. Dissolve in *heptane* and dilute to 50.0 ml with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

Chromatographic system

- a capillary column 30 m x 0.8 mm, packed with fused silica coated with macrogol 20000 (film thickness 0.5 µm),
- temperature:
- column. 170°, then raised at the rate of 3° per minute to 230°,
- inlet port and detector 250°,
- flame ionization detector,
- flow rate: 1.3 ml per minute,
- split ratio of 1:100.

System suitability when using the mixture of calibrating substances in Table 1 or Table 3:

Inject reference solutions (a) and (b). The resolution between the peaks due to methyl oleate and methyl stearate in the

chromatogram obtained with reference solution (a) is not less than 1.8 and the theoretical plates for the peak due to methyl stearate is not less than 30,000. In the chromatogram obtained with reference solution (b), the signal-to-noise ratio for the peak due to methyl myristate is not less than 5.0.

System suitability when using the mixture of calibrating substances in Table 2.

Inject reference solutions (a) and (b). The resolution between the peaks due to methyl caprylate and methyl caprate in the chromatogram obtained with reference solution (a) is not less than 4.0 and the theoretical plates for the peak due to methyl caprate is not less than 15,000. In the chromatogram obtained with reference solution (b), the signal-to-noise ratio for the peak due to methyl caproate is not less than 5.0.

Assessment of chromatograms. Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

Qualitative analysis. Identify the peaks in the chromatogram obtained with reference solution (c) (isothermal operating conditions or linear temperature programming).

When using isothermal operating conditions, the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 1, 2 or 3.

Table 1 – Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system it is recommended that the component with the longest chain length of the mixture under examination to be added to the calibrating mixture, when qualitative analysis is done using calibration curves)

Mixture of following substances	Composition (per cent m/m)
Methyl laurate	5
Methyl myristate	5
Methyl palmitate	10
Methyl stearate	20
Methyl arachidate	40
Methyl oleate	20

Table 2 – Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture under examination to be added to the calibrating mixture, when qualitative analysis is done using calibration curves)

Mixture of following substances	Composition (per cent m/m)
Methyl caproate	10
Methyl caprylate	10
Methyl caprate	20
Methyl laurate	20
Methyl myristate	40

Table 3 – Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture under examination to be added to the calibrating mixture, when qualitative analysis is done using calibration curves)

Mixture of following substances	Composition (per cent m/m)
Methyl myristate	5
Methyl palmitate	10
Ethyl stearate	15
Methyl arachidate	20
Ethyl oleate	20
Methyl eicosenaote	10
Methyl behenate	10
Methyl lignocerate	10

Measure the reduced retention time (t'_R) of each peak in the chromatogram obtained with reference solution (a).

t'_R is the retention time measured from the solvent peak and not from the time of injection. Plot the straight line:

$$\log (t'_R) = f(\text{equivalent chain length})$$

The logarithms of t'_R of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as 'equivalent chain lengths'; the equivalent chain length is the length of the theoretical saturated chain that would have the same t'_R as the fatty acid to be identified. For example, linoleic acid has the same t'_R as the theoretical saturated fatty acid having 18.8 carbon atoms.

Identify the peaks in the chromatogram obtained with the test solution by means of the straight line and the reduced retention times. Equivalent chain lengths are given in Table 4.

Table 4– Equivalent chain lengths (this value, which is to be calculated using calibration curves, is given as an example for a column of macrogol 20000)

Fatty Acid	Equivalent chain length
Caproic acid	6.0
Caprylic acid	8.0
Capric acid	10.0
Lauric acid	12.0
Myristic acid	14.0
Palmitic acid	16.0
Palmitoleic acid	16.3
Margaric acid	17.0
Stearic acid	18.0
Oleic acid	18.3
Linoleic acid	18.8
Gamma-linolenic acid	19.0
Alpha-linolenic acid	19.2
Arachidic acid	20.0
Eicosenoic acid	20.2
Arachidonic acid	21.2
Behenic acid	22.0
Erucic acid	22.2
12-Oxostearic acid	22.7
Ricinoleic acid	23.9
12-Hydroxystearic acid	23.9
Lignoceric acid	24.0
Nervonic acid	24.2

Quantitative analysis. In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except that of the solvent, is set at 100 per cent. The content of a constituent is calculated by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent m/m.

Method B. *This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.*

Test solution. Introduce 0.1 g of the substance under examination into a 10-ml centrifuge tube with a screw cap. Dissolve with 1 ml of *heptane* and 1 ml of *dimethyl carbonate* and mix vigorously under gentle heating (50-60°). Add, while still warm, 1 ml of a 1.2 per cent w/v solution of *sodium* in *anhydrous methanol*, prepared with the necessary precautions, and mix vigorously for about 5 minutes. Add 3 ml of *water* and mix vigorously for about 30 seconds. Centrifuge for 15 minutes at 1500 rpm. Inject 1 µl of the organic phase.

Reference solutions and assessment of chromatograms. Where there is no specific method in the individual monograph, proceed as described under Method A.

Chromatographic system

- a capillary column 30 m x 0.25 mm, packed with fused silica coated with macrogol 20000 (film thickness 0.25 µm),
- temperature:

column	time (min.)	temperature (°)
	0-15	100
	15-36	100-225
	36-61	225,

- injection port and detector 250°,
- flame ionization detector,
- flow rate: 0.9 ml per minute,
- split ratio of 1:100.

Method C. *This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroepoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.*

Test solution. Dissolve 0.1 g of the substance under examination in 2 ml of a 2 per cent w/v solution of *sodium hydroxide* in *methanol* in a 25-ml conical flask and boil under a reflux condenser for 30 minutes. Add 2.0 ml of *boron trifluoride solution* through the condenser and boil for 30 minutes. Add 4 ml of *heptane* through the condenser and boil for 5 minutes. Cool and add 10.0 ml of *sodium chloride solution*, shake for about 15 seconds and add a quantity of *sodium chloride solution* such that the upper phase is brought into the neck of the flask. Collect 2 ml of the upper phase, wash with 3 quantities, each of 2 ml of *water* and dry over *anhydrous sodium sulphate*.

Reference solutions and assessment of chromatograms. Where there is no specific method in the individual monograph, proceed as described under Method A.

2.3.51. 2-Ethylhexanoic Acid

Determine by gas chromatography (2.4.13).

Internal standard solution. Dissolve 100 mg of *3-cyclohexylpropionic acid* in *cyclohexane* and dilute to 100 ml with *cyclohexane*.

Test solution. To 0.3 g of the substance under examination, add 4.0 ml of a 33 per cent v/v solution of *hydrochloric acid*.

Shake vigorously for 1 minute with 1.0 ml of the internal standard solution. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

Reference solution. Dissolve 75 mg of *2-ethylhexanoic acid* in the internal standard solution and dilute to 50.0 ml with the same solution. To 1.0 ml of this solution, add 4.0 ml of a 33 per cent v/v solution of *hydrochloric acid.* Shake vigorously for 1 minute. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

Chromatographic system

- a wide-bore fused-silica column 10 m x 0.53 mm, coated with macrogol 20 000 2-nitroterephthalate (film thickness 1.0 µm),
- temperature:

column	time (min.)	temperature (°)
	0-2	40
	2 - 7.3	40→200
	7.3 - 10.3	200,

- Inlet port at 200° and detector at 300°,
- flame ionization detector,
- flow rate: 10 ml per minute, using nitrogen as carrier gas.

Inject 1 µl of the reference solution. The test is not valid unless the resolution between the peaks corresponding to 2-ethylhexanoic acid (first peak) and the internal standard is not less than 2.0.

Inject 1 µl of the reference solution and the test solution.

Calculate the per cent content of 2-ethylhexanoic acid from the expression:

$$\frac{A_T \times I_R \times m_R \times 2}{A_R \times I_T \times m_T}$$

Where, A_T = Area of the peak corresponding to 2-ethylhexanoic acid in the chromatogram obtained with the test solution,

A_R = Area of the peak corresponding to 2-ethylhexanoic acid in the chromatogram obtained with the reference solution,

I_T = Area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

I_R = Area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,

m_T = Mass of the substance under examination in the test solution, in grams,

m_R = Mass of 2-ethylhexanoic acid in the reference solution, in grams.

2.3.52. Assay of Folic Acid

NOTE—The following procedure is provided for the estimation of folic acid as an ingredient of Pharmacopoeial preparations containing other active constituents.

Solvent mixture. Prepare as directed under Mobile Phase. Adjust to a pH of 7.0, and bubble nitrogen through the solution for 30 minutes before use.

Internal Standard Solution. Weigh accurately 25 mg of *methylparaben* and dissolve in 2.0 ml of *methanol* and dilute to 50.0 ml with solvent mixture and mix.

Test solution. Weigh accurately the substance under examination (containing about 1.0 mg of folic acid) and add 4.0 ml of *Internal Standard Solution* and dilute to 50.0 ml with solvent mixture and mix.

Reference solution (a). Weigh accurately 12 mg of *Folic Acid RS*, and dissolve in 2.0 ml of *ammonium hydroxide Solution* and dilute to 50.0 ml with solvent mixture and mix.

Reference solution (b). To 2.0 ml of *Reference solution (a)*, add 2.0 ml of *Internal Standard Solution* and dilute to 25.0 ml with solvent mixture and mix.

Chromatographic System

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane chemically bonded to porous silica,
- mobile phase: a mixture of 650 ml of a buffer solution prepared by dissolving 2.0 g of *monobasic potassium phosphate* in *water*, add 12.0 ml of a 25 per cent v/v solution of *tetrabutylammonium hydroxide* in *methanol*, add 7.0 ml of *3 M phosphoric acid*, and 240 ml of *methanol*. Cool and adjusted to pH 7.0 with either *3 M phosphoric acid* or *6 M ammonium hydroxide* and dilute to 1000.0 ml with *water*, mix and filter through a 0.45-µm filter,
  (*NOTE — The methanol-to-water ratio may be varied by up to 3 percent and the pH may be increased up to 7.15 to achieve better separation.*)
- flow rate: 1 ml per minute,
- spectrometer set at 280 nm,
- injection volume: 10 µl.

Inject reference solution (b) and the test solution. The relative retention times are about 0.8 for folic acid and 1.0 for methylparaben.

Calculate the content of $C_{19}H_{19}N_7O_6$ in µg.

2.3.53. Ammonium

Unless otherwise prescribed, use Method A.

Method A

Dissolve a quantity of the substance under examination in 14 ml *water* in a test-tube, make alkaline, if necessary by

addition of *dilute sodium hydroxide solution* and dilute to 15 ml with *water*. To the solution, add 0.3 ml of *alkaline potassium tetraiodomercurate solution*. Prepare a standard by mixing 10 ml of *ammonium standard solution* (1 ppm NH_4) with 5 ml of *water* and 0.3 ml of *alkaline potassium tetraiodomercurate solution*. Stopper the test-tube. After 5 minutes, any yellow colour in the test solution is not more intense than that of the standard.

Method B

Dissolve the specified quantity of finely powdered substance under examination in 1 ml of *water* in 25 ml jar fitted with stopper. Add 0.3 g of *heavy magnesium oxide*. Stopper the jar immediately after placing a piece of silver manganese paper 5 mm^2 wetted with a few drops of *water*, under propylene cap. Swirl, avoiding projections of liquid, and allow to stand at 40° for 30 minutes. If the silver manganese paper shows a grey colour, it is not more intense than that of a standard prepared at the same time and in the same manner using the specified volume of *ammonium standard solution*, 1 ml of *water* and 0.3 g of *heavy manganese oxide*.

2.3.54. Assay of Alpha Tocopherol

NOTE—The following procedure is provided for the determination of tocopherol as an ingredient.

Hydrogenator. A suitable device for low-pressure hydrogenation may be assembled as follows. Arrange in a rack or in clamps two conical centrifuge tubes, connected in series by means of glass and inert plastic tubing and suitable stoppers of glass, polymer, or cork (avoiding all use of rubber). Use one tube for the blank and the other for the assay specimen. Arrange a gas-dispersion tube so that the hydrogen issues as bubbles at the bottom of each tube. Pass the hydrogen first through the blank tube and then through the specimen tube.

Procedure. Pipette into a suitable vessel 25 ml of the final washed ether solution of the unsaponifiable fraction obtained as directed for When Tocopherol is present under procedure in the Vitamin A Assay, and evaporate to about 5 ml, remove the remaining ether in a stream of inert gas or by vacuum without applying heat. Dissolve the residue in sufficient alcohol to give an expected concentration of about 0.15 mg of alpha tocopherol per ml. Pipette 15 ml into a 50-ml centrifuge tube, add about 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in the Hydrogenator, using hydrogen that has been passed through alcohol in a blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test solution. 1 ml aliquot of the solution free from the solvent by evaporation, dissolving the residue in 1 ml of chloroform, and adding 10 ml of antimony dichloride. (*NOTE—If a blue color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.*)

Pipet 2 ml of the supernatant into a glass-stopper, opaque flask, add 1.0 ml of a 1 in 500 solution of *ferric chloride* in *dehydrated alcohol* and begin timing the reaction, preferably with a stop watch. Add immediately 1.0 ml of a 1 in 200 solution of 2,2 -bipyridine in *dehydrated alcohol*, mix with swirling, add 21.0 ml of *dehydrated alcohol*, close the tube, and shake vigorously to ensure complete mixing. When about 9½ minutes have elapsed from the beginning of the reaction, transfer part of the mixture to one of a pair of matched 1-cm spectrophotometer cells. After 10 minutes, accurately timed, following the addition of the ferric chloride-dehydrated alcohol solution, determine the absorbance at 520 nm, with a suitable spectrophotometer, (2.4.7) using *dehydrated alcohol* as the blank. Perform a blank determination with the same quantities of the same reagents and in the same manner, but using 2 ml of *dehydrated alcohol* in place of the 2 ml of the hydrogenated solution. Subtract the absorbance determined for the blank from that determined for the assay specimen, and designate the difference as A_D.

Calculate the alpha tocopherol content, in mg, in the assay specimen taken by the formula:

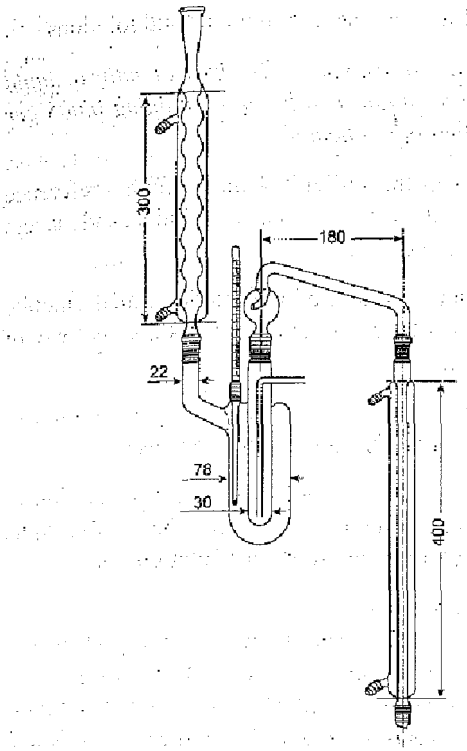
$$= \frac{30.2\,A_D}{LC_D}$$

in which A_D is the corrected absorbance; L is the length, in cm, of the absorption cell; C_D is the content of the assay specimen in the alcohol solution employed for the measurement of absorbance, expressed as g, capsules, or tablets per 100 ml.

NOTE— The absorbance of the blank may be reduced, and the precision of the determination thereby improved, by purification of the dehydrated alcohol that is used throughout the assay. Purification may be accomplished by the addition of a few crystals (about 0.02 per cent) of potassium permanganate and of a few pellets of potassium hydroxide to the dehydrated alcohol, and subsequent redistillation.

2.3.55. Limit Test for Fluorides

Introduce into the inner tube of the apparatus (see Figure 2.3.55-1) the prescribed quantity of the substance under examination, 0.1 g of acid-washed *sand* and 20 ml of a mixture of equal volumes of *sulphuric acid* and *water*. Heat the jacket containing *tetrachloroethane* maintained at its boiling point (146°). Heat the steam generator and distil, collecting the



(*Dimensions in millimetres*)
Fig. 2.3.55-1: Apparatus for limit test for fluorides

distillate in a 100-ml volumetric flask containing 0.3 ml of *0.1 M sodium hydroxide* and 0.1 ml of *phenolphthalein solution*. Maintain a constant volume (20 ml) in the tube during distillation and ensure that the distillate remains alkaline, adding *0.1 M sodium hydroxide* if necessary. Dilute the distillate to 100.0 ml with *water* (test solution). Prepare a standard in the same manner by distillation, using 5 ml of *fluoride standard solution (10 ppm F)* instead of the substance under examination. Into two glass-stoppered cylinders introduce 20 ml of the test solution and 20 ml of the standard and 5 ml of *aminomethylalizarindiacetic acid reagent*.

After 20 minutes, any blue colour in the test solution (originally red) is not more intense than that in the standard.

2.3.56. Assay of Calcium Pantothenate

NOTE — The following liquid chromatographic procedures are provided for the estimation of calcium pantothenate as an active pharmaceutical ingredient in pharmaceutical dosage forms. While conducting these procedures, protect solutions from light, with the use of low-actinic glassware, use the appropriate IP Reference Substances (IPRS).

Assay. Determine by liquid chromatography (2.4.14).

Method A and Method B can be used to determine calcium pantothenate in:

- Oil- and Water-Soluble Vitamins Capsules
- Oil- and Water-Soluble Vitamins Tablets
- Oil- and Water-Soluble Vitamins with Minerals Capsules
- Oil- and Water-Soluble Vitamins with Minerals Tablets
- Water-Soluble Vitamins Capsules
- Water-Soluble Vitamins Tablets
- Water-Soluble Vitamins with Minerals Capsules
- Water-Soluble Vitamins with Minerals Tablets

Method A

NOTE — This procedure involves the extraction of calcium pantothenate from the formulation by the internal standard solution.

Unless specified in the individual monographs, internal standard solution, reference solution, test solution, and reagent solutions are prepared as follows:

Internal standard solution. Dissolve 80 mg of *p-hydroxybenzoic acid* in 3 ml of *ethanol (95 per cent)*. Add 50 ml of *water* and 7.1 g of *dibasic sodium phosphate* and dilute to 1000 ml with *water*, adjusted to pH 6.7 with *orthophosphoric acid*.

Test solution (for capsules). Disperse a quantity of the mixed contents of capsules containing 60 mg of Calcium Pantothenate in 100.0 ml of internal standard solution, centrifuge and use the supernatant liquid.

Test solution (for tablets). Transfer a quantity of the powdered tablets containing 15 mg of Calcium Pantothenate in a centrifuge tube, add 25.0 ml of the internal standard solution, shake vigorously for 10 minutes, centrifuge and use the supernatant liquid.

Reference solution. A 0.06 per cent w/v of *calcium pantothenate IPRS* in internal standard solution

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a 0.1 per cent v/v solution of *orthophosphoric acid* in *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

The relative retention time with reference to *p*-hydroxybenzoic acid for pantothenic acid is about 0.5.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{32}CaN_2O_{10}$.

Method B

NOTE — This procedure involves dissolving an accurately weighed quantity of the sample containing 10 mg of Calcium Pantothenate in 10 ml of methanol and dilute to 250.0 ml with water.

Unless specified in the individual monographs, reference solution, test solution, and reagent solutions are prepared as follows:

Test solution (for capsules). Transfer a quantity of the mixed contents of capsules containing 10 mg of Calcium Pantothenate to a 250-ml volumetric flask, add 10 ml of *methanol*, swirl to disperse and dilute to volume with *water*.

Test solution (for tablets). Transfer a quantity of the powdered tablets containing 10 mg of Calcium Pantothenate to a 250-ml volumetric flask, add 10 ml of *methanol*, swirl to disperse and dilute to volume with *water*.

Reference solution (a). A 0.025 per cent w/v solution of *calcium pantothenate IPRS* in *water* (*NOTE – Use the solution within four weeks and store in refrigerator*).

Reference solution (b). Dilute 4.0 ml of reference solution (a) to 25.0 ml with *water*.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 90 volumes of a buffer solution prepared by dissolving 5 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.5 with *orthophosphoric acid* and 10 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 25 µl.

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{18}H_{32}CaN_2O_{10}$.

Method C

This procedure can be used to determine calcium pantothenate in:

— Oil- and Water-Soluble Vitamins oral solutions

— Oil- and Water-Soluble Vitamins with minerals oral solutions

— Water-Soluble Vitamins with minerals oral solutions

NOTE — This procedure involves dissolve a quantity of liquid sample in water to obtain a solution containing 0.008 per cent v/v of calcium pantothenate.

Unless specified in the individual monographs, reference solution, system suitability solution, test solution, and reagent solutions are prepared as follow.

Test solution. Dilute a volume of oral solution with the mobile phase to obtain a solution containing 0.008 per cent w/v of Calcium Pantothenate.

Reference solution (a). A 0.008 per cent w/v solution of *calcium pantothenate IPRS* in the mobile phase.

Reference solution (b). A 0.008 per cent w/v solution of *racemic panthenol IPRS* in the mobile phase. Mix equal volumes of the solution with reference solution (a).

Chromatographic system
- a stainless steel column 10 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 97 volumes of *0.2 M sodium dihydrogen phosphate* and 3 volumes of *methanol*, adjusted to pH 3.2 with *1.7 M orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to panthenol and calcium pantothenate is not less than 1.5 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{32}CaN_2O_{10}$.

Method D

This procedure can be used to determine calcium pantothenate in:

— Active pharmaceutical ingredients

NOTE — This method involves the dissolution of the sample directly into water.

Unless specified in the individual monographs, reference solution, system suitability solution, test solution, and reagent solutions are prepared as follows.

Test solution. Dissolve 50 mg of the substance under examination in *water* and dilute to 100.0 ml with *water*.

Reference solution (a). A 0.05 per cent w/v solution of *calcium pantothenate IPRS* in *water*.

Reference solution (b). A solution containing 0.01 per cent w/v of *calcium pantothenate IPRS* and 0.05 per cent w/v, each of, *beta alanine IPRS, sodium D-pantoate IPRS* and *pantolactone IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 98 volumes of a buffer solution prepared by dissolving 3.2 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 5.5 with *1 M sodium hydroxide* and 2 volumes of *acetonitrile,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 10 µl.

Name	Relative retention time
Beta alanine	0.3
Pantoic acid	0.6
Pantothenic acid	1.0
Pantolactone	1.9

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to pantothenic acid and pantoic acid is not less than 5.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{32}CaN_2O_{10}$.

2.4. PHYSICAL AND PHYSICOCHEMICAL METHODS

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2.4.1. Appearance of Solution

Clarity of Solution

Special Reagents

Standard Suspension. Dissolve 1.0 g of *hydrazine sulphate* in sufficient *water* to produce 100.0 ml and set aside for about 6 hours. To 25.0 ml of this solution add 25.0 ml of a 10.0 per cent w/v solution of *hexamine*, mix well and allow to stand for 24 hours. Keep in a glass container with a smooth internal surface in which the suspension does not adhere to the glass. Store in this manner, the suspension is stable for about 2 months.

Prepare the *standard suspension* by diluting 15 ml of the well-mixed suspension to 1000 ml with *water*. The *standard suspension* should be used within 24 hours of preparation.

Opalescence Standards. Prepare opalescence standards by mixing aliquots of the *standard suspension* with water as indicated in Table 1. Each opalescence standard should be shaken well before use.

Table 1

Opalescence Standard	Standard Suspension (ml)	Water (ml)
OS1	5.0	95.0
OS2	10.0	90.0
OS3	30.0	70.0
OS4	50.0	50.0

Method. Transfer to a flat-bottom test-tube of neutral glass, 15 to 25 mm in diameter, a suitable volume of the solution under examination such that the test-tubes is filled to a depth of 40 mm. Into another matched test-tube add the same volume of the freshly prepared *opalescence standard*. After 5 minutes, compare the contents of the test-tubes against a black background by viewing under diffused light down the vertical axis of the tubes.

Clarity or opalescence

Express the degree of opalescence in terms of the opalescence standard. A liquid is considered clear if its clarity is the same as that of *water* or of the solvent used for preparing the solution under examination or if its opalescence is not more than that of opalescence standard OS1.

Colour of Solution

Special Reagents

Ferric Chloride Colorimetric Solution (FCS). Dissolve about 46 g of *ferric chloride hexahydrate* in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to produce 1000 ml. Pipette 10 ml of this solution into a 250-ml iodine flask, add 15 ml of *water*, 3 g of *potassium iodide* and 5 ml of *hydrochloric acid* and allow the mixture to stand for 15 minutes. Dilute with 100 ml of *water* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using 0.5 ml of *starch solution*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.02703 g of $FeCl_3,6H_2O$. Adjust the final volume of the solution by the addition of enough of the mixture of *hydrochloric acid* and *water* so that each ml contains 0.045 g of $FeCl_3,6H_2O$.

The solutions should be stored protected from light and standardised before use.

Cobaltous Chloride Colorimetric Solution (CCS). Dissolve about 60 g of *cobaltous chloride* in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to produce 1000 ml. Pipette 5 ml of this solution into a 250 ml iodine flask, add 5ml of *hydrogen peroxide solution (10 volume)* and 15 ml of *sodium hydroxide solution*, boil for 10 minutes, cool and add 2 g of *potassium iodide* and 60 ml of *dilute sulphuric acid*. Dissolve the precipitate by gentle shaking, if necessary, and titrate the liberated iodine with *0.1 M sodium thiosulphate* using 0.5 ml of *starch solution*, added towards the pink end-point, as indicator. Carry out a blank titration.

1ml of *0.1 M sodium thiosulphate* is equivalent to 0.02379 g of $CoCl_2,6H_2O$. Adjust the final volume of the solution by the addition of enough of the above mixture of *hydrochloric acid* and *water* so that each ml contains 0.0595 g of $CoCl_2,6H_2O$.

Cupric Sulphate Colorimetric Solution (CSS). Dissolve about 63 g of *cupric sulphate* in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to produce 1000 ml. Pipette 10 ml of this solution into a 250 ml iodine flask, add 40 ml of *water*, 4 ml of *acetic acid*, 3 g of *potassium iodide*, and 5 ml of *hydrochloric acid* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using 0.5 ml of *starch solution*, added towards the pale brown end-point, as indicator. Carry out a blank titration.

1ml of *0.1 M sodium thiosulphate* is equivalent to 0.02497g of $CuSO_4,5H_2O$. Adjust the final volume of the solution by the addition of enough of the above mixture *hydrochloric acid* and *water* so that each ml contains 0.0624 g of $CuSO_4,5H_2O$.

Reference Solution. Prepare by mixing the volumes of *colorimetric solutions* and *hydrochloric acid (1 per cent w/v HCl)* as indicated in Table 2.

NOTE — Reference solutions must be prepared immediately before use from the Colorimetric solutions which may be stored in refrigerator.

Method

Transfer to a flat bottom test tube of neutral glass 15 to 25 mm in diameter, a suitable volume of a liquid been examined such that the test tube is filled to a depth of 40 mm. Into another matched test tube add the same volume of *water* or of the solvent used for preparing the solution being examined or of the reference solution stated in the individual monograph. Compare the colours in diffused light viewing vertically against a white background.

Colourless Solution. A solution is considered colourless if it has the same appearance as *water* or the solvent used for preparing the solution or is not more intensely coloured than *reference solution BS8*.

Table 2

Colour of reference solution	Reference solution	FCS (ml)	CCS (ml)	CSS (ml)	Hydrochloric Acid (1 per cent w/v HCl) (ml)
Yellow	YS1	24.0	6.0	0	70.0
	YS2	18.0	4.5	0	77.5
	YS3	12.0	3.0	0	85.0
	YS4	6.0	1.5	0	92.5
	YS5	3.2	0.8	0	96.0
	YS6	1.6	0.4	0	98.0
	YS7	0.8	0.2	0	99.0
Greenish Yellow	GYS1	24.0	0.5	0.5	75.0
	GYS2	14.0	0.1	0.1	85.8
	GYS3	8.5	0.05	0.05	91.4
	GYS4	5.0	0.05	0.05	94.9
	GYS5	5.8	0.05	0.05	194.1
	GYS6	2.9	0.05	0.05	197.0
	GYS7	2.9	0.05	0.05	397.0
Brownish Yellow	BYS1	24.0	10.0	4.0	62.0
	BYS2	18.0	7.5	3.0	71.5
	BYS3	12.0	5.0	2.0	81.0
	BYS4	6.0	2.5	1.0	90.5
	BYS5	3.0	1.5	0.5	95.0
	BYS6	1.5	0.8	0.2	97.5
	BYS7	1.0	0.4	0.1	98.5
Brown	BS1	22.5	22.5	18.0	37.0
	BS2	15.0	15.0	12.0	58.0
	BS3	11.2	11.2	9.0	68.6
	BS4	7.5	7.5	6.0	79.0
	BS5	3.7	3.7	3.0	89.6
	BS6	1.5	1.5	1.2	95.8
	BS7	0.8	0.8	0.6	97.8
	BS8	0.4	0.4	0.2	99.0
Red	RS1	10.0	20.0	0	70.0
	RS2	7.5	15.0	0	77.5
	RS3	5.0	10.0	0	85.0
	RS4	3.8	7.6	0	88.5
	RS5	2.5	5.0	0	92.5
	RS6	1.3	2.6	0	96.1
	RS7	0.5	1.0	0	98.5

2.4.2. Atomic Absorption Spectrometry

This technique is based on the fact that when atoms, ions or ion complexes of an element in the ground state are atomised in a flame, they absorb light at the characteristic wavelength of that element. If the absorption process takes place in the flame under reproducible conditions, the absorption is proportional to the number of absorbing atoms.

The measurement of the absorption of radiation by the atomic vapour of the element generated from a solution of that element is the basis of atomic absorption spectrometry. The determination is carried out at the wavelength of one of the absorption lines of the element concerned. The assay is done by comparing the absorbance of the test solution with that of the reference preparation.

Apparatus

An atomic absorption spectrophotometer consists of an emission source that provides the characteristic spectral line of the element such as a hollow-cathode discharge lamp, a monochromator to select the required resonance line, a system for introducing the sample solution into a flame and a detector system.

Since the radiation to be absorbed by the element in the test solution is usually of the same wavelength as that of its emission line, the element in the hollow-cathode lamp is the same as the element to be determined and usually a different lamp is used for each element.

The method of introducing the substance to be analysed depends on the type of atomic generator used. In flame atomic absorption, the sample is nebulised and *water* is the solvent of choice for preparing the test and reference solutions. Organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame. In furnace atomic absorption, the sample may be introduced as a solution in *water* or in an organic solvent.

The atomic vapour may also be generated outside the spectrophotometer as in the case of mercury vapour generator or hydride vapour generator.

Methods

The manufacturer's instructions for the operation of the instrument should be strictly followed.

Unless otherwise directed in the individual monograph, one or the other of the following methods may be used. In Method A, measurements are made by comparison with solutions containing a known amount of the element being analysed by means of a calibration graph and in Method B comparison is made by means of progressive addition of the reference solution of the element being analysed.

Method A – Prepare the solution of the substance under examination (test solution) as directed in the monograph. Prepare not fewer than three standard solutions of the element to be determined, covering the concentration range recommended by the manufacturer of the instrument for the element to be determined and including the expected value in the test solution. Any reagent used in the preparation of the test solution should be added to the standard solutions in the same concentration. After calibrating the instrument as directed above, introduce each standard solution into the flame three times, and record the steady reading, washing the apparatus thoroughly with *water* after each introduction. Between each measurement a blank solution should be aspirated and the reading should be allowed to return to zero level. If a furnace is used, it is fired between readings.

Prepare a calibration curve by plotting the mean of each group of three readings against the concentration of the reference solution and determine the concentration of the element to be determined from the calibration graph.

Method B – Place in each of not fewer than three similar volumetric flasks equal volumes of the test solution as directed in the monograph. Add to all but one of these flasks a measured amount of the specified standard solutions containing steadily increasing amounts of the element being determined. Dilute the contents of each flask to the required volume with *water*. After calibrating the spectrometer as directed above, record the reading of each solution three times. Plot the mean of the readings against concentration of a graph the axis of which intersect at zero added element and zero reading. Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axis represents the concentration of the element being determined in the solution of the substance under examination.

It is advisable to make a stock solution of higher concentration for the reference substance and then dilute it successively to get standard solutions of different concentrations. Care should be taken to avoid manual errors while making dilutions.

NOTE — For the purpose of this Appendix, water refers to deionised purified water distilled immediately before use.

Calcium Solution AAS. Dissolve 1.001 g of *calcium carbonate*, previously dried to constant weight at 105°, in 25 ml of *1M hydrochloric acid*, boil to remove carbon dioxide and add sufficient *water* to produce 1000 ml.

Calcium Solution AAS contains 400 µg of Ca in 1 ml.

Iron Solution AAS. Dilute sufficient quantity of *iron standard solution* with *0.2 M nitric acid* to contain 4 µg of Fe in 1 ml.

Lithium Solution AAS. Dissolve 1.064 g of *lithium carbonate* in 5 ml of *hydrochloric acid* and add sufficient *water* to produce 100 ml.

Lithium Solution AAS contains 2 mg of Li in 1 ml.

Magnesium Solution AAS. Dissolve 5.275 g of *magnesium nitrate* in 16 ml of *dilute nitric acid* and dilute to 500.0 ml with *water*. Magnesium Solution AAS contains 1000 µg of mg in 1 ml.

Potassium Solution AAS. Dissolve 1.144g of *potassium chloride*, previously dried to constant weight at 130°, in sufficient freshly distilled *water* to produce 1000 ml.

Potassium Solution AAS contains 600 µg of K in 1 ml.

Silver Solution AAS. Dissolve 0.790 g of *silver nitrate* in sufficient *water* to produce 1000 ml. Further dilute 1 volume of the resulting solution to 100 volumes with *water*.

Silver solution AAS contains 5 µg of Ag in 1 ml.

Sodium Solution AAS. Dissolve 0.5084 g of *sodium chloride*, previously dried to constant weight at 130°, in sufficiently freshly distilled *water* to produce 1000 ml.

Sodium Solution AAS contains 200 µg of Na in 1 ml.

Strontium Solution AAS. Dissolve 1.685 g of *strontium carbonate* in 10 ml of a 50 per cent v/v solution of *hydrochloric acid* and add sufficient *water* to produce 1000 ml.

Strontium Solution AAS contains 1 mg of Sr in 1 ml.

2.4.3. Atomic Emission Spectrometry

This technique is used to determine the concentration of certain metallic ions by measuring the intensity of emission of light at a particular wavelength by the vapour of the element generated from the substance.

The measurement of the intensity of one of the emission lines of the atomic vapour of the element generated from a solution of that element is the basis of atomic emission spectrometry. The determination is carried out at the wavelength corresponding to this emission line of the element concerned. The assay is done by comparing the intensity of emission from the test solution with that from reference preparations with known concentrations of the element to be determined.

Apparatus

An atomic emission spectrophotometer consists of an atomic generator of the element to be determined (such as flame, plasma, arc etc), a monochromator and a detector. If a flame is used for generating the vapour, *water* is the usual solvent for preparing the test and reference solutions. Organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

Methods

The manufacturer's instructions for the operation of the instrument should be strictly followed. The spectrometer should be operated at the prescribed wavelength setting. Introduce a blank solution into the atomic generator and adjust the instrument reading to zero. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable reading.

Unless otherwise directed in the individual monograph, one or the other of the following methods may be used. In Method A, measurements are made by comparison with solutions containing a known amount of the element being analysed by means of a calibration graph and in Method B comparison is made by means of progressive addition of the reference solution of the element being analysed.

Method A – Prepare the solution of the substance under examination (test solution) as directed in the monograph. Prepare not fewer than three standard solutions of the element to be determined, covering the concentration range recommended by the manufacturer of the instrument for the element to be determined and including the expected value in the test solution. Any reagent used in the preparation of the test solution should be added to the standard solutions in the same concentration. After calibrating the instrument as directed above, introduce each standard solution into the flame three times, and record the steady reading, washing the apparatus thoroughly with *water* after each introduction. Between each measurement a blank solution should be aspirated and the reading should be allowed to return to zero level. If a furnace is used, it is fired between readings.

Prepare a calibration curve by plotting the mean of each group of three readings against the concentration of the reference solution and determine the concentration of the element to be determined from the calibration graph.

Method B – Place in each of not fewer than three similar volumetric flasks equal volumes of the test solution as directed in the monograph. Add to all but one of these flasks a measured amount of the specified standard solutions containing steadily increasing amounts of the element being determined. Dilute the contents of each flask to the required volume with *water*. After calibrating the spectrometer as directed above, record the reading of each solution three times. Plot the mean of the readings against concentration of a graph the axes of which intersect at zero added element and zero reading. Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axis represents the concentration of the element being determined in the solution of the substance under examination.

2.4.4. Flame Photometry

The following methods are based on the measurement of intensity of spectral lines emitted by elements such as sodium, potassium, calcium, etc. The substance containing the element is dissolved in an appropriate solvent (usually water) and subjected to excitation in a flame of appropriate temperature and composition.

Apparatus

Several instruments of suitable selectivity are available. The manufacturer's instructions for the operation of the instrument should be strictly followed.

Methods

Unless otherwise directed in the individual monograph, one or the other of the following methods may be used. In Method A, measurements are made by comparison of sample solutions with solutions containing a known amount of the element being analysed. Method B is suitable for samples that contain very small quantities of the element to be analysed or where there is interference from other elements.

Method A – Prepare a series of standard solutions containing the element to be determined in increasing concentration within the concentration range recommended for the particular instrument used. Choose the appropriate filters or adjust the monochromator to select the wavelength prescribed in the monograph. Spray *water* into the flame and adjust the galvanometer reading to zero. Spray the most concentrated standard solution into the flame and adjust the sensitivity so that a full-scale deflection of the galvanometer is recorded. Again spray water into the flame and when the galvanometer reading is constant readjust it to zero. Spray each standard solution into the flame three times, recording the steady galvanometer readings obtained and washing the apparatus thoroughly with *water* after each spraying. Prepare a calibration curve by plotting the mean of each group of three readings against the concentration. Prepare the solution of the substance under examination as prescribed in the monograph and adjust the strength, if necessary, to bring it into the range of concentration recommended for the instrument used. Spray the solution into the flame three times, recording the galvanometer readings and washing the apparatus thoroughly with *water* after each spraying. Using the mean of the galvanometer readings, determine the concentration of the element being examined from the calibration curve. To confirm the concentration thus obtained, repeat the operations with a standard solution of the same concentration as that of the solution under examination.

Method B – Place in each of not fewer than three similar volumetric flasks equal volumes of the solution of the substance under examination prepared as prescribed in the monograph. Add to all but one of the flasks a measured amount of the prescribed standard solution to produce a series of solutions containing regularly increasing amounts of the element to be determined. Dilute the contents of each flask to the required volume with *water*.

Prepare the flame photometer in the manner described under Method A, using *water* for the adjustment to zero and the solution with the largest amount of added element to adjust the sensitivity so that full scale deflection galvanometer is recorded. Examine each solution three times and plot the mean of the readings against concentration on a graph whose origin or zero reading represents zero concentration of the added element. Extrapolate the straight line joining the points until it meets the extrapolated concentration axis at a point on the negative side. The distance between this point and the intersection of the axis represents the concentration of the element in the solution being examined.

Calcium Solution FP. Dissolve 1.001 g of *calcium carbonate*, previously dried to constant weight at 105°, in 25 ml of *1M hydrochloric acid*, boil to remove carbon dioxide and add sufficient *water* to produce 1000 ml.

Calcium Solution FP contains 400 µg of Ca in 1 ml.

Lithium Solution FP.

Dissolve 1.064 g of *lithium carbonate* in 5 ml of *hydrochloric acid* and add sufficient *water* to produce 100 ml.

Lithium Solution FP contains 2 mg of Li in 1 ml.

Potassium Solution FP. Dissolve 1.144 g of *potassium chloride*, previously dried to constant weight at 130°, in sufficient freshly distilled *water* to produce 1000 ml.

Potassium Solution FP contains 600 µg of K in 1 ml.

Sodium Solution FP. Dissolve 0.5084 g of *sodium chloride*, previously dried to constant weight at 130°, in sufficiently freshly distilled *water* to produce 1000 ml.

Sodium Solution FP contains 200 µg of Na in 1 ml.

2.4.5. Fluorimetry

This procedure uses the measurement of the intensity of the fluorescent light emitted by the substance under examination in relation to that emitted by a given reference standard.

Apparatus

A simple filter fluorimeter or a more sophisticated spectrophotofluorimeter may be used but the latter is superior for analytical purposes on account of wavelength selectivity, accuracy, precision and convenience.

Operate the instrument strictly in accordance with the manufacturer's instructions.

Method

Dissolve the substance under examination in the solvent prescribed in the individual monograph, transfer the solution to the cell or the tube of the spectrofluorimeter or fluorimeter and illuminate it with an excitant light beam of the nominal wavelength prescribed in the monograph and as nearly monochromatic as possible.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence.

For quantitative analysis, introduce into the apparatus the solvent or the mixture of solvents used to dissolve the substance under examination and set the instrument to zero. Introduce the prescribed standard solution and adjust the sensitivity of the instrument so that the reading is close to the maximum. If the adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Finally introduce the solution of the substance under examination and record the intensity of fluorescence. Calculate the concentration, c_x of the substance in the solution to be examined, using the expression:

$$c_x = \frac{I_x \times c_s}{I_s}$$

where, c_x = concentration of the solution to be examined,

c_s = concentration of the standard solution,

I_x = intensity of the light emitted by the solution to be examined, and

I_s = intensity of light emitted by the standard solution.

If the intensity of the fluorescence is not directly proportional to the concentration, the measurement may be effected using a calibration curve. In some cases, measurement can be made with reference to a fixed standard such as a fluorescent glass or a solution of stated concentration of *quinine* in *0.05 M sulphuric acid* or of *fluorescein* in *0.1 M sodium hydroxide*. In such cases, the concentration of the substance under examination must be determined using a calibration curve previously prepared under the same conditions.

2.4.6. Infrared Absorption Spectrophotometry

A. Infrared spectrophotometry

Apparatus

An infrared spectrophotometer for recording the spectra in the infrared region consists of an optical system capable of providing the monochromatic light in the region of 4000 cm^{-1} to 625 cm^{-1} (about 2.5 µm to 16 µm) and the means of measuring the quotient of the intensity of the transmitted light and the incident light. Fourier transform infrared spectrophotometers that are replacing the conventional dispersive instruments use polychromatic radiation and calculate the spectrum in the frequency domain from the original data by Fourier transformation.

For recording infrared spectra, the instrument should comply with the following test for resolution.

Control of resolution performance. For instruments having a monochromator, record the spectrum of a polystyrene film approximately 35 µm in thickness. The difference x (see Fig. 2.4.6-1) between the percentage transmittance at the transmission maximum A at 2870 cm^{-1} (3.48 µm) and that at the transmission minimum B at 2849.5 cm^{-1} (3.51 µm) must be greater than 18. The difference y between the percentage transmittance at the transmission maximum C at 1589 cm^{-1} (6.29 µm) and that at the transmission minimum D at 1583 cm^{-1} (6.32 µm) must be greater than 10.

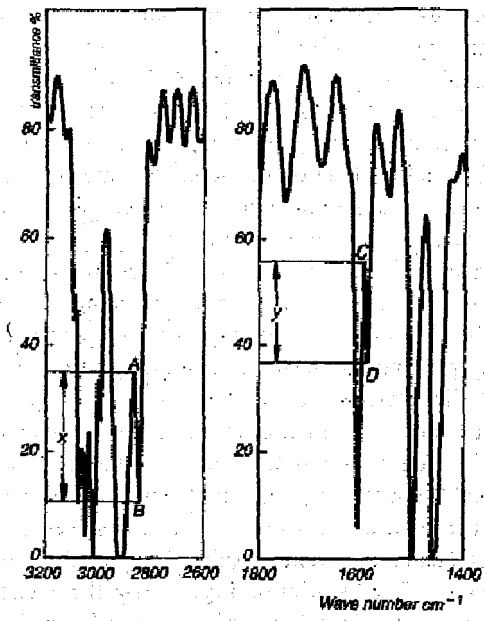


Fig. 2.4.6-1: Typical spectrum of polystyrene used to verify the resolution performance

For Fourier-transform instruments, use suitable instrument resolution with the appropriate apodisation prescribed by the manufacturer. The resolution is checked by suitable means, for example by recording the spectrum of a polystyrene film approximately 35 µm in thickness. The difference between the absorbances at the absorption minimum at 2870 cm^{-1} and the absorption maximum at 2849.5 cm^{-1} is greater than 0.33. The difference between the absorbances at the absorption minimum at 1589 cm^{-1} and the absorption maximum at 1583 cm^{-1} is greater than 0.08.

Verification of the wave-number scale. The wave-number scale may be verified using a polystyrene film, which has

transmission minima (absorption maxima) at the wave-numbers (in cm^{-1}) shown in the Table.

Table: Transmission minima and acceptable tolerances of a polystyrene film

Transmission minima (cm^{-1})	Acceptable tolerance (cm^{-1})	
	Monochromator instruments	Fourier-transform instruments
3060.0	±1.5	±1.0
2849.5	±2.0	±1.0
1942.9	±1.5	±1.0
1601.2	±1.0	±1.0
1583.0	±1.0	±1.0
1154.5	±1.0	±1.0
1028.3	±1.0	±1.0

Preparation of sample. A sample of the substance under examination may be prepared in one of the following ways.

(a) **For normal recording.** *Liquids* — Examine a liquid as a thin film held between two plates or in a cell of suitable path-length constructed of material transparent to infrared radiation in the region to be examined.

Liquids or solids prepared as solutions — Prepare a solution in a suitable solvent and use a concentration and path-length to give a satisfactory spectrum over a sufficiently wide wavelength range. Absorption due to solvent should be compensated for by placing in the reference beam a similar cell containing the solvent used; it should be noted that absorption bands due to the substance under examination that coincide with strong solvent absorption will not be recorded. Suitable concentrations of the solute will vary with the substance being examined but typical concentrations are 1 per cent to 10 per cent at 0.5 to 0.1 mm path-length.

Solids — Examine a solid after dispersion in a suitable liquid (mull) or solid (potassium halide disc), as appropriate.

Mulls — Triturate 1 to 5 mg of the substance with the minimum amount of *liquid paraffin* or other suitable liquid to give a smooth creamy paste. Compress a portion of the mull between two suitable plates.

Discs — Triturate about 1 mg of the substance with approximately 300 mg of dry, finely powdered *potassium bromide IR* or *potassium chloride IR*, as directed. These quantities are usually suitable for a disc 13 mm in diameter. Grind the mixture thoroughly, spread it uniformly in a suitable die and compress under vacuum at a pressure of about 800 MPa. Commercial dies are available and the manufacturer's instructions should be strictly followed. Mount the resultant disc in a suitable holder in the spectrophotometer. Several factors, such as inadequate or excessive grinding, moisture or other impurities in the halide carrier, may give rise to unsatisfactory discs. A disc should be rejected, if visual inspection shows lack of uniformity or if the transmittance at about 2000 cm^{-1} (5 μm) in the absence of a specific absorption band is less than 75 per cent without compensation. If the other ingredients of tablets, injections, or other dosage forms are not completely removed from the substance being examined, they may contribute to the spectrum.

(b) For recording by multiple reflection. When directed in the individual monograph, prepare the sample by one of the following methods.

Solutions — Dissolve the substance in the appropriate solvent under the conditions described in the individual monograph. Evaporate the solution on a thallium bromo-iodide plate or any other suitable plate.

Solids — Place the substance on a thallium bromo-iodide plate or any other suitable plate in a manner giving uniform contact.

Identification by reference substances. Unless otherwise directed in the individual monograph, prepare the substance under examination and the Reference Substance in the form of discs dispersed in *potassium bromide IR* or *potassium chloride IR* and record the spectra between 4000 cm^{-1} and 625 cm^{-1} (2.5 μm to 16 μm) under the same operational conditions. The absorption maxima in the spectrum obtained with the substance under examination correspond in position and relative intensity to those in the spectrum obtained with the Reference Substance. When the spectra recorded in the solid state show differences in the positions of the absorption maxima, treat the substance under examination and the Reference Substance in the same manner so that they crystallise or are produced in the same form, or otherwise proceed as directed in the monograph, and then record the spectra.

Identification by reference spectra. Unless otherwise directed in the individual monograph or on the reference spectrum prepare the substance as a disc in a dispersion of *potassium bromide IR* and record the spectrum from 2000 cm^{-1} to 625 cm^{-1} (5 μm to 16 μm); in some cases the spectrum should be scanned from 4000 cm^{-1} (2.5 μm). The spectrum should be scanned using the same instrumental conditions as were used to ascertain compliance with the requirement for resolution. To allow for possible differences in wavelength calibration between the instrument on which the reference spectrum was obtained and that on which the spectrum of the substance is being recorded, suitable reference absorption maxima of a polystyrene spectrum are superimposed on the reference spectrum or the specimen should be checked with the specimen of polystyrene. These will normally occur at about 2851 cm^{-1} (3.51 μm), 1601 cm^{-1} (6.25 μm) and 1028 cm^{-1} (9.73 μm), but when there is interference with any of these maxima by a band

in the spectrum of the substance under examination, alternative reference maxima are marked on the reference spectrum. Similar reference maxima should be superimposed on the spectrum of the substance. With reference to these maxima of polystyrene, the positions and relative intensities of the absorption bands of the substance should conform to those of the reference spectrum. When comparing the two spectra, care should be taken to allow for the possibility of differences in resolving power between the instrument on which the reference spectrum was prepared and the instrument being used to examine the substance. A reference spectrum of a polystyrene film recorded on the same instrument as the reference spectrum of the substance is included for assessing these differences. The greatest variations due to differences in resolving power are likely to occur in the region between 4000 cm^{-1} and 2000 cm^{-1} (2.5 μm to 5 μm).

Reference spectra. Infrared Reference Spectra of the articles of the pharmacopoeia are provided in Chapter 3.1.

B. Near-infrared spectrophotometry

The near-infrared (NIR) spectral range extends from about 780 nm to about 2,500 nm (from about 12,800 cm^{-1} to about 4,000 cm^{-1}). NIR spectra are dominated by C-H, N-H, O-H and S-H overtone resonances and combinations of fundamental vibrational modes. NIR bands are much weaker than the fundamental mid-IR vibrations from which they originate. Because molar absorptivities in the NIR range are low, radiation typically penetrates several millimeters into materials, including solids. Furthermore, many materials such as glass are relatively transparent in this region.

Measurements can be made directly on *in situ* samples, in addition to standard sampling and testing procedures. Physical as well as chemical information, both qualitative and quantitative, is available from NIR spectra. However, direct comparison of the spectrum obtained with the substance under examination with a reference spectrum of a chemical reference substance, as used in infrared absorption spectrophotometry, is not appropriate. Suitable validated mathematical treatment of the data is required.

Measurements in the NIR region are influenced by many chemical and physical factors as described below; reproducibility and relevance of results depend on control of these factors and measurements are usually valid only for a defined calibration model.

Apparatus. All NIR measurements are based on passing light through or into a sample and measuring the attenuation of the emerging (transmitted, scattered or reflected) beam. Spectrophotometers for measurement in the NIR region consist of a suitable light source, a monochromator or interferometer. Common monochromators are acousto-optical tuneable filters (AOTF), gratings or prisms. High intensity light sources such as quartz or tungsten lamps or similar are used. The tungsten lamp light source can be highly stabilised. Therefore many NIR instruments have the single-beam design. Silicon, lead sulphide, indium arsenide, indium gallium arsenide, mercury cadmium telluride (MCT) and deuterated triglycine sulphate are commonly used detector materials. Conventional cuvette sample holders, fibre-optic probes, transmission dip cells and spinning or traversing sample holders are a few common sampling devices. The selection is based on the intended application, paying particular attention to the suitability of the sampling system for the type of sample to be analysed. Suitable data processing and evaluation units are usually part of the system.

Measurement methods

Transmission mode. Transmittance (T) is a measure of the decrease in radiation intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and detector. The arrangement is analogous to that in many conventional spectrophotometers and the result can be presented directly in terms of transmittance (T) or/and absorbance (A).

$$T = \frac{I}{I_o}$$

where, I_0 = intensity of incident radiation,
I = intensity of transmitted radiation.

$$A = -\log_{10} T = \log_{10}\left(\frac{I}{T}\right) = \log_{10}\left(\frac{I_o}{I}\right)$$

Diffuse reflection mode. The diffuse reflection mode gives a measure of reflectance (R), the ratio of the intensity of light reflected from the sample (I) to that reflected from a background or reference reflective surface (I_r). NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed by vibrational combinations and overtone resonances of the analyte species present in the sample. Non-absorbed radiation is reflected back from the sample to the detector. NIR reflectance spectra are typically obtained by calculating and plotting log ($1/R$) versus the wavelength or wavenumbers.

$$R = \frac{I}{I_r}$$

where, I = intensity of light diffusively reflected from the sample,
I_r = intensity of light reflected from the background or reference reflective surface.

$$A_R = \log_{10}\left(\frac{I}{R}\right) = \log_{10}\left(\frac{I_r}{I}\right)$$

Transflection mode. This mode is a combination of transmittance and reflectance. In the measurement of transflectance (T^*) a mirror or a diffuse reflectance surface is used to reflect the radiation transmitted through the sample a second time and thus doubling the pathlength. Non-absorbed radiation is reflected back from the sample to the detector.

$$T^* = \frac{I}{I_T}$$

where, I_T = intensity of transflected radiation, without sample,

I = intensity of transmitted and reflected radiation measured with the sample.

$$A^* = \log_{10}\left(\frac{I}{T^*}\right)$$

Sample preparation/presentation

Transmission mode. The measurement of transmittance (T) is dependent on a background transmittance spectrum for its calculation. A background reference can be air, an empty cell, and a solvent blank or in special cases a reference sample. The method generally applies to liquids, diluted or undiluted, dispersions, solutions and solids. For transmittance measurements of solids, a suitable sample accessory is to be used. The samples are examined in a cell of suitable path length (generally 0.5-4 mm), transparent to NIR radiation, or by immersion of a fibre optic probe of a suitable configuration, which yields a spectrum situated in a zone of transmission compatible with the specifications of the apparatus and appropriate for the intended purpose.

Diffuse reflection mode. This method generally applies to solids. The sample is examined in a suitable device. Care must be taken to make the measuring conditions as reproducible as possible from one sample to another. When immersing a fibre optic probe in the sample, care must be taken in the positioning of the probe to ensure that it remains stationary during the acquisition of the spectra and that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references are ceramic tiles, perfluorinated polymers and gold. Other suitable materials may be used. Only spectra measured against a background possessing the same optical properties can be directly compared with one another. The particle size, water of hydration and state of solvation must be taken into consideration.

Transflection mode. A reflector is placed behind the sample so as to double the pathlength. This configuration can be adopted to share the same instrument geometry with reflectance and fibre optic probe systems where the source and the detector are on the same side of the sample. The sample is examined in a cell with a mirror or a suitable diffusive reflector, made either of metal or of an inert substance (for example titanium dioxide) not absorbing in the NIR region.

Factors affecting spectral response

Factors that can affect the spectral response are sample temperature, moisture and solvent residues, sample thickness, sample optical properties, polymorphism and the age of samples. Care must be taken to ensure that samples for NIR analysis are representative of those used for calibration. Suitable adjustments may be made to the test conditions in order to cope with these factors.

Control of instrument performance. Use the apparatus according to the manufacturer's instructions and carry out the prescribed verification at regular intervals, according to the use of the apparatus and the substances to be tested.

Verification of the wavelength scale (Except for filter apparatus). Verify the wavelength scale employed, generally in the region between about 780 nm and about 2,500 nm (about 12,800 cm^{-1} to about 4,000 cm^{-1}) or in the intended spectral range using one or more suitable wavelength standards which have characteristic maxima or minima within the range of wavelengths to be used. Dichloromethane or a mixture of rare-earth oxides are suitable reference materials. Take one spectrum with the same spectral resolution used to obtain the certified value, and measure the position of at least 3 peaks distributed over the range used. Acceptable tolerances are ± 1 nm at 1,200 nm, ± 1 nm at 1,600 nm and ± 1.5 nm at 2,000 nm (± 8 cm^{-1} at 8,300 cm^{-1}, ± 4 cm^{-1} at 6,250 cm^{-1} and ± 4 cm^{-1} at 5,000 cm^{-1}). For the reference material used, apply the tolerance for the nearest wavelength (wavenumber) from the above for each peak used. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow water-vapour line at 7299.86 cm^{-1} or a narrow line from a certified material. For rare-earth oxides, NIST 1920 (a) is the most appropriate reference.

Measurement in transmission mode. *Dichloromethane* may be used at an optical path length of 1.0 mm. It has characteristic sharp bands at 1,155 nm, 1,366 nm, 1,417 nm, 1,690 nm, 1,838 nm, 1,894 nm, 2,068 nm and 2,245 nm. The bands at 1,155 nm, 1,417 nm, 1,690 nm and 2,245 nm are used for calibration. Other suitable standards may also be used.

Measurement in diffuse reflection (reflectance) mode. A mixture of dysprosium, holmium and erbium oxides (1+1+1 by mass) or other certified material may be used. This reference material exhibits characteristic peaks at 1,261 nm, 1,681 nm and 1,935 nm. If it is not possible to use external solid standards and if measurements of diffuse reflection are carried out in cells or if fibre optic probes are used, a suspension of 1.2 g of

titanium dioxide in about 4 ml of *dichloromethane*, vigorously shaken, is used directly in the cell or probe. The spectrum is recorded after 2 min. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2,500 nm (16 cm^{-1} at 4,000 cm^{-1}). Measurement is made of the position of at least 3 peaks distributed over the range used. The acceptance tolerances are given under Verification of the wavelength scale. For the reference material used, apply the tolerance for the nearest wavelength (wavenumber) for each peak used.

Verification of the wavelength repeatability (Except for filter apparatus). Verify the wavelength repeatability using suitable standards. The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer.

Verification of photometric linearity and response stability. Verification of photometric linearity is demonstrated with a set of transmission or reflection standards with known values of transmittance or reflectance in percentage. For reflectance measurements, carbon-doped polymer standards are available. At least 4 reference standards in the range of 10.0 to 90.0 per cent such as 10.0 per cent, 20.0 per cent, 40.0 per cent and 80.0 per cent with respective absorbance values of 1.0, 0.7, 0.4 and 0.1 are used. If the system is used for analytes with absorbances higher than 1.0, 2.0 per cent and/or 5.0 per cent standard is added to the set. Plot the observed absorbance values against the reference absorbance values and perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept.

Spectra obtained from reflectance standards are subject to variability due to the difference between the experimental conditions under which they were factory-calibrated and those under which they are subsequently put to use. Hence, the percentage reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an "absolute" calibration for a given instrument. But as long as the standards do not change chemically or physically and the same reference background is used as was used to obtain the certified values, subsequent measurements of the same standards under identical conditions including precise sample positioning give information on long-term stability of the photometric response. A tolerance of ± 2 per cent is acceptable for long-term stability; this is only necessary if spectra are used without pre-treatment.

Verification of photometric noise. Determine the photometric noise using a suitable reflectance standard, for example white reflective ceramic tiles or reflective thermoplastic resins (for example, PTFE). Scan the reflection standard over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. The value is approximately twice the standard deviation. The photometric noise is consistent with the specification of the spectrophotometer.

Qualitative analysis

Establishment of reference spectra. Record the spectra of a suitable number of batches of the substance which have been fully tested according to established specifications and which exhibit the variation typical for the substance to be analysed (for example, manufacturer, physical form, particle size). The set of spectra represents the information for identification and characterisation that defines the similarity border for that substance and is the entry for that substance in the spectral collection used to identify the substance. The number of substances in a collection depends on the specific application. All spectra should have the same: (a) spectral range and number of data points; (b) technique of measurement; (c) data pre-treatment.

If sub-groups are created, the above criteria are applied independently for each group. The collection of spectra may be represented in different ways defined by the mathematical technique used for identification. These may be: (a) all individual spectra representing the substance; (b) a mean spectrum of each batch of substance; (c) if necessary, a description of the variability within the substance spectra. (d) electronic raw data for the preparation of the spectral collection must be archived.

Pre-treatment of data. In many cases, and particularly for reflection mode spectra, some form of mathematical pretreatment of the spectrum may be useful before the development of a classification or calibration model. The aim can be to reduce baseline variations, to reduce the impact of known variations that are interfering in the subsequent mathematical models, or to compress data before use. Typical methods are multiplicative scatter correction (MSC), the Kubelka-Munk transforms, spectral compression techniques that may include windowing and noise reduction and the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended. In some cases spectra may also be normalised, for example against the maximum absorbance, the mean absorbance or the integrated absorbance area under the spectrum.

Caution must be exercised when performing any mathematical transformation, as artefacts can be introduced or essential information (important with qualification methods) can be lost. An understanding of the algorithm is required and in all cases the rationale for the use of transform must be documented.

Data evaluation. Direct comparison of the spectrum of the substance under investigation is made with the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable

algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification. The reliability of the algorithm chosen for a particular application has to be validated. For example, correlation coefficient, the sum of squared residuals or the distance using cluster analysis must comply with the acceptance limits defined in the validation procedure.

Validation of the database. Parameters to be taken into account are specificity and robustness.

Quantitative analysis

Establishment of a spectral data base for a calibration model. Calibration is the process of constructing a mathematical model to relate the response from an analytical instrument to the properties of the samples. Any calibration algorithm that can be clearly defined in an exact mathematical expression and gives suitable results can be used. Record spectra of a suitable number of samples with known values of the content throughout the range to be measured (for example, content of water). Wavelengths used in the calibration model can be compared to the known bands of the analyte and those of the matrix to verify that the bands of the analyte of interest are being used by the calibration. Establish the calibration model with about two-thirds of the measured samples. Compare the remaining one-third of the measured samples with the database. All samples must give quantitative results within a precision interval as defined by the intended purpose of the method. Correct quantification must be demonstrated in the presence of variations in the matrix within the specified range. Multiple linear regression (MLR), partial least squares (PLS) and principal component regression (PCR) are commonly used. For PLS or PCR calibrations, the coefficients or the loadings can be plotted and the regions of large coefficients compared with the spectrum of the analyte. Raw data for the preparation of the calibration model must be archived, without data pretreatment.

Pre-treatment of data. Data pre-treatment can be defined as the mathematical transformation of the NIR spectral data to enhance spectral features and/or remove or reduce unwanted sources of variation prior to the development of the calibration model. Many suitable algorithms for data pre-treatment and calibration exist. The selection is based on the suitability for the intended use. Wavelength selection may enhance the efficiency of calibration models such as MLR (for example, in particle-size determination). It is useful to delete certain ranges of the wavelength scale in some cases, for example in the determination of water of hydration. Wavelength compression may be applied to the data.

Validation. The conventional analytical performance characteristics should be considered for demonstrating the validation of NIR methods. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method.

Ongoing model evaluation. NIR models validated for use should be subjected to ongoing performance evaluation and monitoring of validation parameters. If discrepancies are found, corrective action will be necessary.

Transfer of databases. When databases are transferred to another instrument, spectral range, number of data points, spectral resolution and other parameters have to be taken into consideration. Further procedures and criteria must be applied to demonstrate that the model remains valid with the new database or new instrument.

2.4.7. Ultraviolet and Visible Absorption Spectrophotometry

Ultraviolet and visible absorption spectrophotometry is the measurement of the absorption of monochromatic radiation by solutions of chemical substances, in the range of 185 nm to 380 nm, and 380 nm to 780 nm of the spectrum, respectively.

The magnitude of the absorption of a solution is expressed in terms of the absorbance, A, defined as the logarithm to base 10 of the reciprocal of transmittance (T) for monochromatic radiation:

$$A = \log_{10}(I_o / I)$$

where I_o is the intensity of the incident radiation I, is the intensity of the transmitted radiation. The absorbance depends on the concentration of the absorbing substance in the solution and the thickness of the absorbing layer taken for measurement.

For convenience of reference and for ease in calculations, the specific absorbance of a 1 per cent w/v solution is adopted in this Pharmacopoeia for several substances unless otherwise indicated, and it refers to the absorbance of a 1 per cent w/v solution in a 1 cm cell and measured at a defined wavelength. It is evaluated by the expression

$$A\,(1\ \text{per cent}, 1\ \text{cm}) = A/cl,$$

where c is the concentration of the absorbing substance expressed as percentage w/v and l is the thickness of the absorbing layer in cm. The value of A (1 per cent, 1 cm) at a particular wavelength in a given solvent is a property of the absorbing substance.

Unless otherwise stated, measure the absorbance at the prescribed wavelength using a path length of 1 cm at 24° to 26°. Unless otherwise stated, the measurements are carried out with reference to the same solvent or the same mixture of solvents.

Apparatus

A spectrophotometer, suitable for measuring in the ultraviolet and visible ranges of the spectrum consists of an optical system capable of producing monochromatic light in the range of 200 nm to 800 nm and a device suitable for measuring the absorbance.

The two empty cells used for the solutions under examination and the reference liquid must have the same spectral characteristics. Where double-beam-recording instruments are used, the solvent cell is placed in the reference beam.

Control of wavelengths

Verify the wavelength scale using the absorption maxima of *holmium perchlorate solution*, the line of hydrogen or deuterium discharge lamp or the lines of a mercury vapour are shown below. The permitted tolerance is ± 1 nm for the range 200 nm to 400 nm and ± 3 nm for the range 400 nm to 600 nm.

241.15 nm (Ho)	404.66 nm (Hg)
253.70 nm (Hg)	435.83 nm (Hg)
287.15 nm (Ho)	486.00 nm (Dβ)
302.25 nm (Hg)	486.10 nm (Hβ)
313.16 nm (Hg)	536.30 nm (Ho)
334.15 nm (Hg)	546.07 nm (Hg)
361.50 nm (Ho)	576.96 nm (Hg)
365.48 nm (Hg)	579.07 nm (Hg)

Control of absorbance

Check the absorbance using suitable filters or solutions of *certified nicotinic acid for calibration* and *certified potassium dichromate for calibration* at the wavelengths indicated in below Table, which gives for each wavelength the exact values and permitted limits of the specific absorbance. The tolerance for the absorbance is ± 0.01.

Use solution of *certified nicotinic acid for calibration* for the control of absorbance at 213 nm and 261 nm, prepared by dissolving 15.0 mg of *certified nicotinic acid for calibration* in *0.1 M hydrochloric acid* and dilute to 1000.0 ml with the same acid.

Use solutions of *certified potassium dichromate for calibration* which has been previously dried to constant weight at 130°, for the control of absorbance at 235 nm, 257 nm, 313 nm and 350 nm, prepared by dissolving 57.0 - 63.0 mg of *certified potassium dichromate for calibration* in *0.005 M sulphuric acid* and dilute to 1000.0 ml with the same acid. For the control of absorbance at 430 nm, dissolve 57.0-63.0 mg of *certified potassium dichromate for calibration* in *0.005 M sulphuric acid* and dilute to 100.0 ml with the same acid.

Table

Wavelength (nm)	Specific absorbance A (1 per cent, 1 cm)	Maximum Tolerance
213	424.4	419.4 to 429.3
235	124.5	122.9 to 126.2
257	144.5	142.8 to 146.2
261	419.2	412.0 to 423.9
313	48.6	47.0 to 50.3
350	107.3	105.6 to 109.0
430	15.9	15.7 to 16.1

Limit of stray light

Stray light may be detected at a given wavelength with suitable filters or solutions; for example, absorbance of a 1.2 per cent w/v solution of *potassium chloride IR* in a 1 cm cell should be greater than 2.0 at about 198 nm when compared with *water* as reference liquid.

Resolution

When stated in a monograph, record the spectrum of 0.02 per cent v/v solution of *toluene* in *hexane UV*. The ratio of the absorbance at the maximum at about 269 nm to that at the minimum at about 266 nm is not less than 1.3 unless otherwise specified in the monograph.

Spectral slit width

When measuring the absorbance at an absorption maximum the spectral slit width must be small compared with the half width of the absorption band otherwise erroneously low absorbance will be measured. Particular care is needed for certain substances and the instrumental slit width used should always be such that further reduction does not result in an increased absorbance reading.

Cells

The absorbances of the cells intended to contain the solution under examination and the reference liquid, when filled with the same solvent should be identical. If this is not the case, an appropriate correction must be applied. The tolerance on the path length of the cells used is ± 0.5 per cent (e.g. ± 0.005 cm for a 1 cm cell). Cells should be cleaned and handled with great care.

Solvents

In measuring the absorbance of a solution at a given wavelength, the absorbance of the reference cell and its contents should not exceed 0.4 and should preferably be less than 0.2 when measured the reference to air at the same wavelength. The solvent in the reference cell should be of the same lot as that used to prepare the solution and must be free from fluorescence at the wavelength of measurement. *Ethanol*

(95 per cent), ethanol, methanol, and cyclohexane, used as solvents, should have an absorbance measured in a 1 cm cell at about 254 nm with reference to *water* not exceeding 0.10.

Determination of absorbance

Unless otherwise directed, measure the absorbance at the prescribed wavelength using a path length of 1 cm at 24° to 26°. If necessary, the path length may be varied provided that compliance with Beer's Law has been shown over the range in question.

A statement in a assay or test of the wavelength at which maximum absorption occurs implies that the maximum occurs either precisely at or within ± 2 nm of the given wavelength. Likewise, a statement in a test of the absorbance, A, at a given wavelength or at the maximum at about a specified wavelength implies that the measured absorbance is within ± 3 per cent of the stated value.

When an assay or test prescribes the use of a Reference Substance, make the spectrophotometric measurements with the solution prepared from the Reference Substance by the official directions and then with the corresponding solution prepared from the substance under examination. Carry out the second measurement as quickly as possible after the first, using the same cell and same experimental conditions.

Unless otherwise specified, the requirements in the monographs for light absorption in the tests and assays apply to the dried or anhydrous material, where a standard is given for loss on drying or content of water, respectively. Similar considerations apply where standards are given for solvent content. In calculating the result, the loss on drying or contents of water or solvent, determined by the method specified in the monograph, are taken in to account.

Derivative spectrophotometry

Derivative spectrophotometry involves the transformation of absorption spectra (zero-order) into first-, second- or higher-order derivative spectra.

A first order-derivative spectrum is a plot of the gradient of the absorption curve (rate of change of the absorbance with wavelength, $dA/d\lambda$) against wavelength.

A second order-derivative spectrum is a plot of the curvature of the absorption spectrum ($d^2A/d\lambda^2$) against wavelength. If the absorbance follows the Beer-Lambert relationship, the second order-derivative at any wavelength λ is related to the concentration by the following expression.

$$d^2A/d\lambda^2 = d^2A\,(1\text{ per cent}, 1\text{ cm}) \times cd/d\lambda^2$$

where, A = the absorbance at wavelength λ,
$A\,(1\text{ per cent}, 1\text{ cm})$ = the specific absorbance at wavelength λ
c = the concentration of the absorbing solute express as a percentage w/v,
d = the thickness of the absorbing layer in cm.

Apparatus

A spectrophotometer complying with the requirements for Control of wavelengths and Control of absorbances described above and equipped with an analog resistance-capacitance differentiation module or a digital differentiator or another means of producing second order-derivative spectra should be used in accordance with the manufacturer's instructions. Some methods of producing second order-derivative spectra lead to a wavelength shift relative to the zero order spectrum and this should be taken into account when necessary. Unless otherwise stated in the monograph, the spectral slit width of the spectrophotometer, where variable, should be set as described under Spectral slit width above. The cells and solvents used should comply with the statements given under Cells and Solvents respectively.

Reagents

The temperatures of all solutions used in the test should not differ by more than 0.5°.

Resolution power

When stated in a monograph, record the second order-derivative spectrum in the range 255 nm to 275 nm of a 0.020 per cent v/v solution of *toluene* and in *methanol* using *methanol* in the reference cell. A small negative extremum (or trough) located between two large negative extrema (or troughs) at about 261 nm and 268 nm should be clearly visible.

Procedure

Prepare the solution of the substance under examination, adjust the various instrument settings as stated in the manufacturer's instructions and calculate the amount of the substance under examination as stated in the monograph.

2.4.8. Boiling Range or Temperature and Distillation Range

The boiling or distilling range of a liquid is the temperature interval, corrected for a pressure of 101.3 kPa within which the liquid, or a specified fraction of the liquid, distils under the conditions specified in the test. The lower limit of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser, and the upper limit is the temperature at which the last drop evaporates from the lowest point in the distillation flask; it may also be the temperature observed when the proportion specified in the individual monograph has been collected.

Apparatus

Use an apparatus consisting of the following:

Distilling flask. A round-bottom distilling flask of 200-ml capacity and having a total length of 17 to 19 cm and an inside neck diameter of 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side-arm 10 to 12 cm long and 5 mm in internal diameter which is at an angle of 70° to 75° with the lower portion of the neck.

Condenser. A straight glass condenser 55 to 60 cm long with a water-jacket about 40 cm long or any other type of condenser having equivalent condensing capacity. The lower end of the condenser may be bent to provide a delivery tube, or it may be connected to a bent adaptor that serves as a delivery tube.

Receiver. A 100-ml cylinder graduated in 1-ml sub-divisions.

Thermometer. An accurately standardised, partial immersion thermometer having the smallest practical sub-divisions (not greater than 0.2°). When placed in position, the stem is located in the centre of the neck and the top of the bulb is just below the bottom of the outlet to the side arm.

Method

If the liquid under examination distils below 80°, cool it to between 10° and 15° before measuring the sample for distillation.

Assemble the apparatus and place in the flask 100 ml of the liquid being examined, taking care not to allow any of the liquid to enter the side-arm. Insert the thermometer and shield the entire heating and flask assembly from external air currents. Add a few pieces of porous material and heat rapidly to boiling using a Bunsen burner (or an electric heater or mantle with arrangement for adjustment of the applied heat) and an asbestos plate pierced by a hole 33 mm in diameter. Record the temperature at which the first drop of distillate falls into the receiver and adjust the rate of heating to obtain a regular distillation rate of 4 to 5 ml per minute. Record the temperature when the last drop of liquid evaporates from the lowest point in the distillation flask or when the specified percentage has distilled over. Correct the observed temperature readings for any variation in the barometric pressure from the normal (101.3 kPa) using the following expression.

$$t_1 = t_2 + K(a - b),$$

where, t_1 = the corrected temperature,

t_2 = the observed temperature,

a = 101.3, when the barometric pressure is measured in kilopascals (kPa), or 760 when measured in torr,

b = the barometric pressure at the time of the determination,

K = the correction factor indicated in Table.

Table – Variation of correction factor with temperature.

Boiling range (°)	K_{kPa}	K_{torr}
Less than 100	0.30	0.040
100 to 140	0.34	0.045
141 to 190	0.38	0.050
191 to 240	0.41	0.055
More than 240	0.45	0.060

2.4.9. Conductivity

The current I (in amperes) flowing in a conductor is directly proportional to the applied electromotive force E (in volts) and inversely proportional to the resistance R (in ohms) of the conductor.

$$I = \frac{E}{R}$$

The conductivity of a solution (K) is the reciprocal of resistivity (ρ) which is defined as the quotient of the electric field and the density of the current (flowing in the conducting solution). The resistance R (in Ω) of a conductor of cross-section S (in cm^2) and length L (in cm) is given by the expression $R = \rho \times L/S$ or $1/K \times L/S$; thus, $K = 1/R \times L/S$ where, L/S corresponds to the ideal cell constant.

The unit of conductivity in the International System is the siemens per metre ($S\ m^{-1}$). What is generally used in expressing the electrical conductivity of a solution is siemens per centimetre ($S\ cm^{-1}$) or microsiemens per centimetre ($\mu S\ cm^{-1}$). The resistivity of a solution is expressed in ohm-centimetres (Ω cm).

Unless otherwise stated, the reference temperature for the expression of conductivity or resistivity is 25°.

Apparatus. The apparatus used is a conductivity meter that measures the resistance of the column of liquid between the electrodes of the immersed conductivity cell (the measuring device). The meter is supplied with alternating current and is equipped with a temperature probe and a temperature compensation device. The generally used conductivity cell contains two parallel platinum electrodes coated with platinum black, each with a surface area S, and separated from the other by a distance L. The electrodes are protected by a glass tube. Other types of cells may also be used.

Procedure

Determination of the cell constant. Use a certified reference material (such as a solution of potassium chloride) with conductivity less than 1500 $S\ cm^{-1}$ and the cell constant shall be within 2 per cent of the given value. A high cell constant is

necessary when solutions of high conductivity are tested. Commonly used conductivity cells have cell constants of the order of 0.1 cm^{-1}, 1 cm^{-1} and 10 cm^{-1}.

Use a certified reference material (such as a solution of potassium chloride) with a conductivity value near the expected value of the solution under examination. Rinse the cell several times with *distilled water* and at least twice with the certified reference material used for the determination of the cell constant of the conductivity cell. Measure the resistance of the conductivity cell using the certified reference material at 25 ± 1°. The cell constant is given by the expression $K = R_{cm} \times K_{cm}$ where, R_{cm} is the measured resistance in megaohms and K_{cm} is the conductivity of the certified reference material solution used, in microsiemens per centimetre.

NOTE — Other certified reference materials may be used especially for cells having a constant of 0.1 cm^{-1}.

The measured constant K of the conductivity cell must be within 5 per cent of the value indicated.

If the cell constant is determined at a temperature other than that indicated for the certified reference material, the conductivity value is calculated from the expression:

$$K_t = K_{cm} \times [1 + \alpha (T - T_{cm})]$$

where, K_t = value of conductivity at the different temperature,

K_{cm} = value of conductivity of the certified reference material,

T = temperature set for calibration,

T_{cm} = temperature indicated for the certified reference material,

α = temperature coefficient for the conductivity value of the certified reference material (0.021 for potassium chloride).

Calibration of meter. Calibration can be done by replacing the conductivity cell with officially certified precision resistors (accurate to ± 0.1 per cent of the stated value) or an equally accurate adjustable resistance device to give a predicted instrument response. Each scale on the meter may have to be calibrated prior to use. The instrument must have a minimum resolution of 0.1 µS cm^{-1}.

Method. After the apparatus has been calibrated with a certified reference material solution, rinse the conductivity cell several times with *distilled water* and at least twice with the aqueous solution under examination. Carry out successive measurements as described in the individual monograph.

Water conductivity. A three-stage method of testing is described for Purified Water and Water for Injections (WFI). Testing at the first stage is usually adequate for Purified Water. Testing at two preliminary stages is given for WFI. If the test conditions and conductivity limits are met at either of these preliminary stages, the water meets the requirements of the test. In such cases proceeding to the third stage may not be necessary. Only in the event of failure at the final stage is the sample to be considered as not complying with the requirements of the test.

Stage 1 of the procedure may alternatively be performed on-line (with suitable modifications of the first step) with instrumentation that has been appropriately calibrated, whose cell constants have been accurately determined, and whose temperature compensation has been disabled. Prior to testing it must be ensured that such instrumentation has been suitably located and fitted in the water system.

Procedure

Stage 1.

1. Measure the temperature of the *water* using a non-temperature compensated conductivity reading. The measurement may be done in a suitable container or as an on-line determination.
2. Using Table 1/ Table 2 find the temperature value that is not greater than the measured temperature and read the corresponding conductivity value that becomes the limit (*Do not interpolate*).
3. If the measured conductivity is not greater than the table value, the *water* meets the requirements of the test. If the conductivity is higher than the table value, proceed with testing at stage 2.

Table 1

Temperature (°)	Purified Water Conductivity (µS cm^{-1})
0	2.4
10	3.6
20	4.3
25	5.1
30	5.4
40	6.5
50	7.1
60	8.1
70	9.1
75	9.1
80	9.7
90	9.7
100	10.2

Hold the stopper firmly in place when vig... begun, invert the flask so as to provide ... care to prevent incompletely burne... liquid. When combustion is ... vigorously for about 5 min... cup top, carefully remo... platinum wire, plac... *water*. Proceed ...

NOTE—S... the sur...

...lents of active ... obtained in ...

...ature of the titrant during the ...

...6. PHENOL IN VACCINES AND ANTISERA

60	–
65	–
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Stage 2.

4. Transfer a sufficient amount of water (100 ml or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and while maintaining it at 25 ± 1°, begin vigorously agitating the sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than 0.1 µS cm^{-1} per 5 minutes, note the conductivity.

5. If the conductivity is not greater than 2.1 µS cm^{-1}, the *water* under examination meets the requirements of the test. If the conductivity is greater than 2.1 µS cm^{-1}, proceed with the testing at stage 3. For Sterile Water for Inhalation/Sterile Water for Injections: For containers with a nominal volume of 10 ml or less, if the conductivity is NMT 25 µS cm^{-1}, the water meets the requirements. For containers with a nominal volume greater than 10 ml, if the conductivity is NMT 5 µS cm^{-1}, the water meets the requirements.

Stage 3.

6. Perform this test within approximately 5 minutes of the conductivity determination in step 5 under stage 2, while maintaining the sample temperature at 25 ± 1°. Add a recently prepared saturated solution of *potassium chloride* to the test sample (0.3 ml per 100 ml of the test sample), and determine the pH (2.4.24) to the nearest 0.1 pH unit.

Using Table 3, determine the conductivity limit at the measured pH value in step 6. If the measured conductivity in step 4 under stage 2 is not greater than the conductivity requirements for the pH determined, the water under examination meets the requirements of the test. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0 to 7.0, the water under examination does not meet the requirements of the test.

Table 3

pH	Conductivity (µS cm^{-1})
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.2
6.5	2.2
6.6	2.2
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

2.4.10. Congealing Range or Temperature

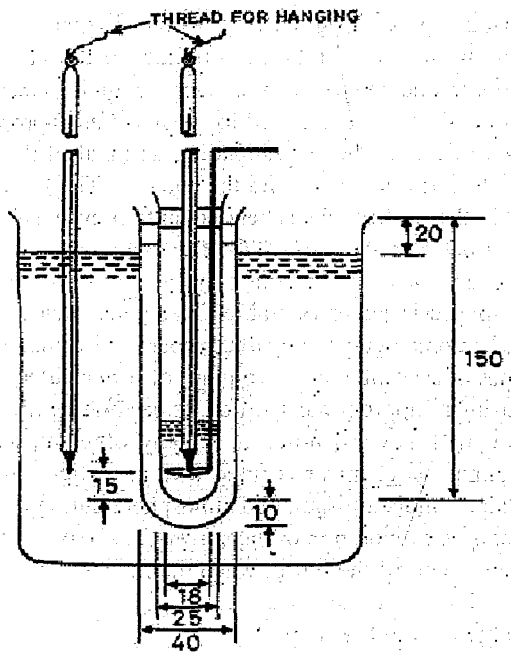
The congealing temperature is that point at which there exists a mixture of the liquid (fused) phase of a substance and a small but increasing proportion of the solid phase. It is distinct from the freezing point which is the temperature at which the liquid and solid phases of a substance are in equilibrium. In certain cases, this may happen over a range of temperatures

The temperature at which a substance solidifies upon cooling is a useful index of its purity if heat is liberated when solidification takes place.

The following method is applicable to substances that melt between –20° and 150°.

Apparatus

A test-tube (about 150 mm x 25 mm) placed inside another test-tube (about 160 mm x 40 mm) the inner tube is closed by a stopper that carries a stirrer and a thermometer (about 175 mm long and with 0.2° graduations) fixed so that the bulb is about 15 mm above the bottom of the tube. The stirrer is made from wire of stainless steel, type 316 or other suitable inert material formed at one end into loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1-litre beaker containing a suitable cooling liquid to within 20 mm of the top. The thermometer is supported in the cooling bath (see Fig. 2.4.10-1).



(Dimensions in mm)

Fig. 2.4.10-1: Apparatus for Determination of Congealing Range or Temperature

Method

Melt the substance, if a solid, at a temperature not more than 20° above its expected congealing point, and pour it into the inner test-tube to a height of 50 to 57 mm. Assemble the apparatus with the bulb of the thermometer immersed half-way between the top and bottom of the sample in the test-tube. Fill the bath to almost 20 mm from the top of the tube with a suitable fluid at a temperature 4° to 5° below the expected congealing point. If the substance is a liquid at room temperature, carry out the determination using bath temperature about 15° below the expected congealing point. When the sample has cooled to about 5° above its expected congealing point stir it continuously by moving the loop up and down between the top and bottom of the sample at a regular rate of 20 complete cycles per minute. If necessary, congealation may be induced by scratching the inner walls of the test-tube with the thermometer or by introducing a small amount of the previously congealed substance under examination. Pronounced supercooling may result in deviation from the normal pattern of temperature changes. If it happens, repeat the test introducing small fragments of the solid substance under examination at 1° intervals when the temperature approaches the expected congealing point.

Record the reading of the thermometer every 30 seconds and continue stirring only so long as the temperature is falling. Stop the stirring when the temperature is constant or starts to rise slightly. Continue recording the temperature for at least 3 minutes after the temperature again begins to fall after remaining constant.

The congealing point will be the mean of not less than four consecutive readings that lie within a range of 0.2°.

2.4.11. Freezing Point

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

Apparatus

The apparatus and its dimensions (in mm) are as shown in Fig.2.4.10-1.

Method

Place a quantity of the liquid or previously melted substance under examination in the inner tube such that the thermometer bulb is well covered and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5° above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with *water* or a saturated solution of *sodium chloride* at a temperature about 5° lower than the approximate freezing point, assemble the apparatus, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. The highest temperature observed during solidification of the substance is regarded as the freezing point of the substance.

Table 2

Temperature (°)	Water for Injections and Sterile Water for Injections/ Inhalation Conductivity ($\mu S\ cm^{-1}$)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Stage 2.

4. Transfer a sufficient amount of water (100 ml or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and while maintaining it at 25 ± 1°, begin vigorously agitating the sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than 0.1 $\mu S\ cm^{-1}$ per 5 minutes, note the conductivity.

5. If the conductivity is not greater than 2.1 $\mu S\ cm^{-1}$, the *water* under examination meets the requirements of the test. If the conductivity is greater than 2.1 $\mu S\ cm^{-1}$, proceed with the testing at stage 3. For Sterile Water for Inhalation/Sterile Water for Injections: For containers with a nominal volume of 10 ml or less, if the conductivity is NMT 25 $\mu S\ cm^{-1}$, the water meets the requirements. For containers with a nominal volume greater than 10 ml, if the conductivity is NMT 5 $\mu S\ cm^{-1}$, the water meets the requirements.

Stage 3.

6. Perform this test within approximately 5 minutes of the conductivity determination in step 5 under stage 2, while maintaining the sample temperature at 25 ± 1°. Add a recently prepared saturated solution of *potassium chloride* to the test sample (0.3 ml per 100 ml of the test sample), and determine the pH (2.4.24) to the nearest 0.1 pH unit.

7. Using Table 3, determine the conductivity limit at the measured pH value in step 6. If the measured conductivity in step 4 under stage 2 is not greater than the conductivity requirements for the pH determined, the water under examination meets the requirements of the test. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0 to 7.0, the water under examination does not meet the requirements of the test.

Table 3

pH	Conductivity ($\mu S\ cm^{-1}$)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.2
6.5	2.2
6.6	2.2
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

2.4.10. Congealing Range or Temperature

The congealing temperature is that point at which there exists a mixture of the liquid (fused) phase of a substance and a small but increasing proportion of the solid phase. It is distinct from the freezing point which is the temperature at which the liquid and solid phases of a substance are in equilibrium. In certain cases, this may happen over a range of temperatures.

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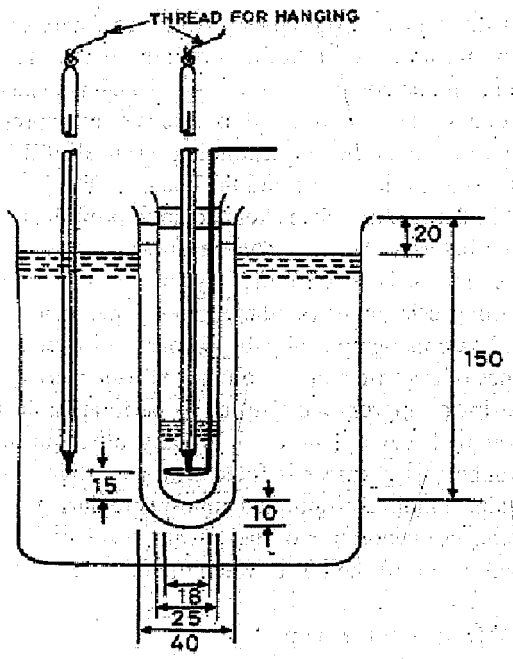


(Dimensions in mm)

Fig. 2.4.10-1: Apparatus for Determination of Congealing Range or Temperature

Method

Melt the substance, if a solid, at a temperature not more than 20° above its expected congealing point, and pour it into the inner test-tube to a height of 50 to 57 mm. Assemble the apparatus with the bulb of the thermometer immersed halfway between the top and bottom of the sample in the test-tube. Fill the bath to almost 20 mm from the top of the tube with a suitable fluid at a temperature 4° to 5° below the expected congealing point. If the substance is a liquid at room temperature, carry out the determination using bath temperature about 15° below the expected congealing point. When the sample has cooled to about 5° above its expected congealing point stir it continuously by moving the loop up and down between the top and bottom of the sample at a regular rate of 20 complete cycles per minute. If necessary, congealation may be induced by scratching the inner walls of the test-tube with the thermometer or by introducing a small amount of the previously congealed substance under examination. Pronounced supercooling may result in deviation from the normal pattern of temperature changes. If it happens, repeat the test introducing small fragments of the solid substance under examination at 1° intervals when the temperature approaches the expected congealing point.

Record the reading of the thermometer every 30 seconds and continue stirring only so long as the temperature is falling. Stop the stirring when the temperature is constant or starts to rise slightly. Continue recording the temperature for at least 3 minutes after the temperature again begins to fall after remaining constant.

The congealing point will be the mean of not less than four consecutive readings that lie within a range of 0.2°.

2.4.11. Freezing Point

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

Apparatus

The apparatus and its dimensions (in mm) are as shown in Fig. 2.4.10-1.

Method

Place a quantity of the liquid or previously melted substance under examination in the inner tube such that the thermometer bulb is well covered and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5° above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with *water* or a saturated solution of *sodium chloride* at a temperature about 5° lower than the approximate freezing point, assemble the apparatus, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. The highest temperature observed during solidification of the substance is regarded as the freezing point of the substance.

2.4.12. Electrophoresis

Electrophoresis is a physical method of analysis based on the migration of electrically charged proteins, colloids, molecules or other particles dissolved or dispersed in an electrolyte solution in the direction of the electrode bearing the opposite polarity when an electric current is passed through it. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Due to differences in the physico-chemical properties, different macromolecules of a mixture migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Separations may be conducted in systems without support phases (such as free solution separation in capillary electrophoresis) or in stabilising media such as thin-layer plates, films or gels.

The electrophoretic mobility is the rate of movement in metre per second of the charged particles under the action of an electric field of 1 volt per metre and is expressed in square metres per volt second. For practical reasons it is given in square centimetres per volt second, $cm^2 V^{-1}S^{-1}$. The mobility is specific for a given electrolyte under precisely determined operational conditions.

Depending on the method used, the electrophoretic mobility is either measured directly or compared with that of a reference substance.

Based upon the type of apparatus used, electrophoretic methods may be divided into two categories, one called free or moving boundary and the other called zone electrophoresis (using a supporting medium).

Moving Boundary Electrophoresis

This method is chiefly employed with substances of high relative molecular weight and low diffusibility. In the method a buffered solution of proteins in a U-shaped cell is subjected to an electric current which causes the proteins to form a series of layers in order of decreasing mobility, which are separated by boundaries. Only a part of the fastest moving protein is physically separated from the other proteins, but examination of the moving boundaries by physical processes such as refractometry or conductimetry helps to locate the boundaries and also provides data for calculation of mobilities and information on the qualitative and quantitative composition of the protein mixture. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

Zone Electrophoresis

This method requires the use of small samples only.

In zone electrophoresis, the sample is introduced as a narrow zone or spot in a column, slab or film of buffer. Migration of the components as narrow zones permits their complete separation. Remixing of the separated zones by thermal convection is prevented by stabilising the electrolyte in a porous matrix such as powdered solid, or a fibrous material such as paper, foil or gel such as agar, starch or polyacrylamide.

The rate of migration depends on four main factors viz. the mobility of the charged particle, the electro-endosmotic flow, the evaporation flow, and the strength of the field. It is necessary to operate under clearly defined experimental conditions and to use, wherever possible, Reference Substances.

Apparatus

The apparatus consists essentially of the following:

(1) An appropriate power source supplying a direct current and provided with means for indicating and controlling either the output voltage or the current consumption as appropriate; the output may be stabilised suitably.

(2) An electrophoresis chamber which is usually rectangular and made of glass or rigid plastic, with two separate troughs, the anodic and the cathodic, containing the electrolyte solution. In one compartment of each trough is immersed an electrode; these are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the two troughs is kept equal to prevent siphoning. Contact between the inner and outer compartments of each double trough is made either by means of "bridges" of electrophoresis paper (suitable filter paper that has been washed chromatographically for 16 hours with a mixture of 20 volumes of *acetone* and 10 volumes of *water*, dried and cut into strips of appropriate size) or by perforating the central portion with several holes or by any other means. The electrophoresis chamber is fitted with an airtight lid which maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is advisable to cool the support.

(3) A support-carrying device suitable for the type of electrophoresis to be carried out.

(a) *For strip electrophoresis* — The supporting strip, previously wetted with the same conducting solution and dipping at each end into that compartment of each trough that does not contain the electrode, is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte, such as a horizontal frame, inverted-V stand or a uniform surface with contact points at suitable intervals.

(b) *For gel electrophoresis* — The device consists essentially of a glass plate over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions are to be taken to avoid condensation of moisture or drying of the solid layer.

(4) A measuring device or means of detection.

Method

Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. Switch off the current and remove the support from the chamber, dry and visualise.

CAUTION — Voltages used in electrophoresis can deliver a lethal shock. The equipment, with the exception of the power supply, should be enclosed in either a grounded metal case or a case of insulating material. High-voltage cables should be such that the central conductor is completely shielded and the base of the apparatus should be grounded metal or contain a grounded metal rim so constructed that any leakage of electrolyte will produce a short-cutting of the power supply before the electrolyte can flow beyond the protective enclosure.

Cellulose Acetate Electrophoresis

Use Method I unless otherwise directed.

Method I

Fill the troughs of the apparatus with the electrolyte solution specified in the monograph. Immerse cellulose acetate foil of suitable dimensions for 5 minutes in the same solution and press the strips dry between filter paper. Apply separately to the foil at points 1 cm from the anode edge and 2.5 cm apart 1 µl of each of the solutions prescribed. Adjust the voltage to that given in the monograph and allow electrophoresis to proceed for the specified time. Press the strips dry and immerse in a solution prepared by dissolving 1 g of *potassium ferricyanide* in 50 ml of *water* and adding 2 ml of a saturated solution of *ferric chloride*. Wash with a 5 per cent v/v solution of *phosphoric acid* until the background is as pale as possible and finally wash with *water*. Examine the electropherogram.

Method II

Fill the troughs of the apparatus with *mixed barbitone buffer pH 8.6*. Use a separate strip of cellulose acetate for each solution prescribed in the monograph and apply either 2.5 µl of the solution as a 10-mm band or, if narrower strips are used, 0.25 µl of the solution per mm of strip width. Apply a suitable electric field such that the most rapid band migrates at least 30 mm. Stain the strips with a 0.5 per cent w/v solution of *naphthalene black 12B* in a mixture of 90 volumes of *methanol* and 10 volumes of *5 M acetic acid* for 5 minutes and then decolorise with a mixture of 90 volumes of *methanol* and 10 volumes of *acetic acid* so that the background is just free of colour. Wash the strips with a mixture of 81 volumes of *methanol* and 19 volumes of *5 M acetic acid* until the background is as transparent as possible. Measure the absorbance of the bands at about 600 nm in an instrument having a linear response over the range of at least 0 to 3 (2.4.7). Calculate the result as the mean of three measurements of each strip.

Polyacrylamide Gel Electrophoresis

Apparatus

This consists of two buffer solution reservoirs made of suitable material such as poly (methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply operating either at constant current or at constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

Method

Degas the solutions before polymerisation and use the gels immediately after preparation.

Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, ensuring that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water* to exclude air and allow to set. A gel is usually formed in about 30 minutes and a sharp interface between the gel and the water layer should be formed. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution.

Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude

the gel. Locate the position of the bands in the electropherogram as prescribed.

Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

This method is used for the qualitative characterisation of proteins in biological preparations, for control of purity, assessments of the homogeneity of proteins and quantitative determinations. It can be adopted for the routine estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins.

Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis using *sodium dodecyl sulphate* (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent *sodium dodecyl sulphate* (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

Reducing conditions. Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulphide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulphide bonds. Complete denaturation and dissociation of proteins by treatment with *2-mercaptoethanol* or *dithiothreitol* (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular-mass standards.

Non-reducing conditions. For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as *2-mercaptoethanol* or DTT, disulphide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. This makes molecular- weight determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

Characteristics of discontinuous buffer system gel electrophoresis

The most popular electrophoretic method for the characterisation of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution that drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel.

Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the *tris (hydroxymethyl) aminomethane* and *glycine*. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular weights.

Method

Commercially available ready-to-use gels may be used for the test.

Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse the slot with *SDS-PAGE running buffer*. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness. Electrophoresis running time and current/voltage may need to vary as described by the manufacturer of the apparatus in order to achieve optimum separation. Check that the dye front is moving into the resolving gel. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

Detection of proteins in gels

Coomassie staining is the most common protein staining method with a detection level of the order of 1 µg to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected.

All the steps in gel staining are done at room temperature with gentle shaking (e.g. on an orbital shaker platform) in any convenient container. Gloves must be worn when staining gels, since fingerprints will stain.

Coomassie staining. Immerse the gel in a large excess of *Coomassie staining solution* and allow to stand for at least 1 hour. Remove the staining solution.

Destain the gel with a large excess of *destaining solution*. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the destaining solution.

NOTE — The acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol and 5 volumes of water for 1 hour before it is immersed in the Coomassie staining solution.

Silver staining. Immerse the gel in a large excess of *fixing solution* and allow to stand for 1 hour. Remove the *fixing solution*, add fresh *fixing solution* and incubate either for at least 1 hour or overnight, if convenient. Discard the *fixing solution* and wash the gel in a large excess of *water* for 1 hour. Soak the gel for 15 minutes in a 1 per cent v/v solution of *glutaraldehyde*. Wash the gel twice for 15 minutes in a large excess of *water*. Soak the gel in fresh *silver nitrate reagent* for 15 minutes, in darkness. Wash the gel three times for 5 minutes in a large excess of *water*. Immerse the gel for about 1 minute in *developer solution* until satisfactory staining has been obtained. Stop the development by incubation in the *blocking solution* for 15 minutes. Rinse the gel with *water*.

Drying of stained SDS polyacrylamide gels

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 10 per cent w/v solution of *glycerol* for at least 2 hours (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 minutes in a 2.0 per cent w/v solution of *glycerol*.

Immerse two sheets of porous cellulose film in *water* and incubate for 5 minutes to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of *water* around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

Molecular weight determination

Molecular weights of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of proteins with precisely known molecular weights blended for uniform staining are available for calibrating gels. They are obtainable in various molecular weight ranges. Concentrated stock solutions of proteins of known molecular weight are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalised migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_f. Construct a plot of the logarithm of the relative molecular weights (M_r) of the protein standards as a function of the R_f values. Note that the graphs are slightly sigmoid. Unknown molecular weights can be estimated by linear regression analysis or interpolation from the curves of log M_r against R_f as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

Validation of the test

The test is not valid unless the proteins of the molecular weight marker are distributed along 80 per cent of the length of the gel and over the required separation range (e.g. the range covering the product and its dimer or the product and its related impurities) the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular weight and the R_f. Additional validation requirements with respect to the solution under test may be specified in individual monographs.

Quantification of impurities

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalisation to the main band using an integrating densitometer. In this case, the responses must be validated for linearity.

2.4.13. Gas Chromatography

Gas Chromatography (GC), also known as Gas Liquid Chromatography (GLC), is a technique for separation of mixtures into components by a process which depends on the redistribution of the components between a stationary phase or support material in the form of a liquid, solid or combination of both and a gaseous mobile phase. It is applicable to substances or their derivatives which are volatilized under the temperature employed. GC is based on mechanisms of adsorption, mass distribution or size exclusion.

Apparatus

The apparatus consists of an injector, a chromatographic column contained in an oven, a detector and a data acquisition system. The carrier gas flows through the column at a controlled rate or pressure and then through the detector.

Injectors. Direct injections of solutions are the usual mode of sample introduction unless otherwise prescribed in the monograph. Injection may be carried out either directly at the head of the column using a syringe or an injection valve, or into a vaporization chamber which may be equipped with a stream splitter.

Injections of vapour phase may also be effected by static or dynamic head space injection systems. Dynamic head space (purge and trap) injection system include a sparging device by which volatile substances from a solution are swept into an adsorbent column maintained a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the adsorbent column.

Static head space injection systems include thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow the volatile components of the sample to reach equilibrium between the non-gaseous phase and the vapour phase. After equilibrium has been established, a pre-determined quantity of the head space vapour from the vial is automatically introduced into the gas chromatograph either by a heated syringe or a transfer line and sample loop.

Stationary Phases. Stationary phases are contained inside the column. A GC column can be:

A) a fused silica capillary column whose wall is coated with the stationary phase.

B) a metallic or glass column packed with inert particles impregnated with the stationary phase.

C) a metallic or glass column packed with solid stationary phase.

A wide range of chemical substances are used as stationary phase in GC. These include polyethylene glycols, high molecular weight esters and amides, hydrocarbons, silicon gums and fluids (polysiloxanes substituted with methyl, phenyl, cyano, vinyl or fluroalkyl groups or mixtures of these) and solid adsorbents like micro porous cross-linked polyaromatic beads, molecular sieves etc.

Capillary columns can be of 0.1 mm to 0.53 mm in internal diameter and 10 meter to 100 meter in length. The liquid or solid stationary phase may be chemically bonded to the inner surface of the tubing with a coating thickness of 0.1 µ to 5.0 µ.

Packed columns, made of glass or metal, are usually 1 meter to 3 meter in length with an internal diameter of 2 mm to 4 mm.

Support materials must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanising prior to coating with liquid phase. Acid washed, flux-calcinated diatomaceous earth is often used as support material. The support materials are available in various particle sizes, the most commonly used ones in the range of 100 to 120 mesh.

Mobile phases. Mobile phases that are employed in GC are inert gases. The commonly used gases are Nitrogen, Hydrogen and Helium. The source of carrier gas can be a pressurized cylinder or a gas generator which can provide a continuous flow of the gas. The purity of the gas should be minimum 99.99 per cent. The gas should pass through a purification panel having suitable filters for the removal of residual moisture, oxygen and hydrocarbons before entering the GC. The pressure and flow rate of the carrier gas should be adjusted to get optimum separation of sample components.

Detectors. Flame ionization detectors are used, unless otherwise mentioned. Additional detectors which may be used include: thermal conductivity, electron capture, nitrogen-phosphorus, flame photometric and mass spectrometric depending upon the purpose of the analysis.

Method. Equilibrate the column, the injector and the detector (flame ionisation, unless otherwise stated in the individual monograph) at the specified temperatures and flow rates until a stable base line is obtained. Prepare the test and reference solutions as prescribed in the monograph. The solutions must be free from solid particles. Using the solution of the reference substance determine experimentally suitable instrument settings and volumes of solutions to be injected to produce an adequate response. Inject the selected volumes of the solutions prescribed in the monograph and record the resulting chromatograms. Repeat the determinations to ensure a consistent response. Determine the peak areas or peak heights corresponding to the peaks of interest. In determinations requiring temperature programming, peak areas should be considered. From the values obtained, calculate the content of the components being determined.

Normalization. Where reference is made to normalization for the estimation of one or more components, the total area of the peak or peaks due to the components is expressed as a percentage of the sum of the areas of all the peaks derived from the substance being examined.

Internal Standard. Where reference is made to internal standard method for the estimation of one or more components, a suitable internal standard should be selected for the purpose. The selected internal standard should not contain any impurity that is likely to interfere in the determination described in the monograph.

Injection volume. Where no injection volume is specified in the monograph, the analyst should select an appropriate volume for their specific application. The volume chosen is dependent on the response of the analyte, the detector used, the efficiency of the column and the overall performance of the chromatographic system. Where a volume is not indicated, 1 µl is usually appropriate; however this should be checked for suitability under the local operating conditions.

Secondary peaks. Reference may be made to a secondary peak. A secondary peak is a peak in the chromatogram other than the principal peak and any peaks due to internal standard, solvent and derivatising agents. Peaks identified as being due to the counter-ion and/or other excipients including preservatives in the material being examined may also be excluded.

Performance

Resolution. Unless otherwise stated in the monograph, the Resolution factor (Rs) between measured peaks on the chromatogram must be greater than 1.0 and is defined by the expression:

$$Rs = \frac{1.18(t_{r2} - t_{r1})}{W_{h1} + W_{h2}}$$

t_{r1} and t_{r2} = retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of 2 adjacent peaks. $t_{r2} > t_{r1}$,

w_{h1} and w_{h2} = peak widths at half height.

A resolution greater than 1.5 corresponds to baseline separation. The values of t_{r1}, t_{r2}, w_{h1} and w_{h2} must be expressed in the same unit of measurement.

Symmetry factor. The symmetry factor or tailing factor of a peak is calculated from the expression:

$$As = \frac{w_{0.05}}{2d}$$

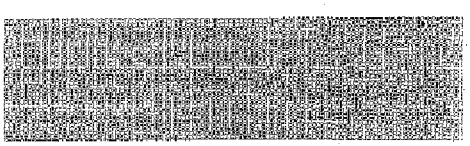
where, $w_{0.05}$ = width of the peak at one-twentieth of the peak height,

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

A value of 1.0 signifies complete or ideal symmetry.

Column efficiency. When column efficiency is stated in the monograph, it is defined in terms of the number of theoretical plates (N) by the expression :

$$N = 5.54 (t_r / w_h)^2$$



where, t_r = retention time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h = width of the peak at half height.

The apparent number of theoretical plates varies with the sample component as well as with the column.

Capacity factor or mass distribution ratio. The capacity factor (k') also known as mass distribution ratio (D_m) is defined as:

$$D_m = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}}$$

$$= Kc\,(V_s/V_m),$$

where, Kc = equilibrium distribution coefficient,

V_s = volume of stationary phase,

V_m = volume of mobile phase.

The capacity factor of a component may be calculated from the chromatogram using the equation:

$$K' = \frac{t_R - t_M}{t_M}$$

where, t_R = retention time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

t_M = time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained peak.

Relative retention. Absolute retention time of a compound may vary depending upon column and analysis conditions. Comparisons are normally made in terms of relative retention (a), which is calculated from the expression:

$$a = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

where, t_{R2} = retention time of the peak of interest,

t_{R1} = retention time of the reference peak,

t_M = hold-up time: time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained peak.

Where the value of t_M is small, the relative retention time (Rr) may be estimated from the expression:

$$Rr = t_{R2}/t_{R1}$$

Signal to noise ratio. The signal-to- noise ratio is determined from the expression:

$$S/N = 2H / h$$

where, H = height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height,

h = range of the background noise in a chromatogram obtained after injection of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

System suitability. Unless otherwise stated in the monograph, the maximum permitted relative standard deviation for replicate injections of the prescribed reference solution does not exceed 2 per cent. This requirement applies only to assays.

Adjustment to chromatographic conditions

Adjustments may be made to the parameters of the test as given below in order to satisfy the system suitability criteria without fundamentally modifying the method.

Stationary phase.

- column length: ± 70 per cent,
- column internal diameter: ± 50 per cent,
- particle size: reduction of not more than 50 per cent, no increase,
- film thickness: - 50 per cent to + 100 per cent.

Flow rate. ± 50 per cent.

Temperature. ± 10 per cent.

Injection volume and Split volume. The injection volume and split volume may be adjusted if detection and repeatability are satisfactory.

Static head – space gas chromatography

Static head-space gas chromatography is a technique suitable for separating and determining volatile compounds present in non-volatile solid or liquid samples. The method is based on the analysis of the vapour phase in equilibrium with the solid or liquid phase.

Apparatus

The apparatus consists of a gas chromatograph connected to a head space sampler intended to introduce the vapour in the

GC column. The sample to be analyzed is introduced into a glass container fitted with a suitable stopper. The container is placed in a thermostatically controlled chamber at a temperature set according to the substance under examination. The sample is held at this temperature long enough to allow equilibrium to be established between the solid or liquid phase and the vapour phase. After the equilibration period, a portion of the vapour phase can be transferred to the GC column either by a heated syringe or with the help of a transfer line and fixed volume loop. Using the reference preparations, determine suitable instrument settings to produce an adequate response.

2.4.14. Liquid Chromatography

Liquid chromatography (LC) is a separation technique based on the difference in the distribution of components between two non-miscible phases in which liquid mobile phase elutes through a stationary phase in a column. The three forms of high performance liquid chromatography most often used are based on mechanism of partition, adsorption and ion exchange.

Ion exchange chromatography, also referred to as ion chromatography, is an analytical technique for the separation and determination of ionic solutes i.e. inorganic cations, inorganic anions, low molecular weight (water soluble) organic acids and bases etc. The separation of ionic solutes takes place on the basis of ion exchange on stationary phases with charged functional groups. The functional groups typically are quaternary ammonium groups for anion exchange and negatively charged groups like sulphonates for cation exchange. The corresponding counter ions are located in vicinity of the functional groups and can be exchanged with other ions of the same charge in the mobile phase. Thus, various ionic components of the sample can be separated based on their differential affinities towards the immobilized stationary and the liquid mobile phase.

The combination of ion exchange columns with conductivity detection with or without chemical suppression represents the most important and popular type of ion chromatography. In the technique with chemical suppression, the conductivity of the ionic mobile phase is suppressed both chemically and electronically. In case of technique without chemical suppression, the conductivity of mobile phase is suppressed only electronically.

In addition, size exclusion and stereochemical interaction phenomena are also used for separation.

Apparatus

A pumping system, an injector, a chromatographic column with or without a column temperature controller, a detector and a data acquisition system (a computer, an integrator or a chart recorder) are the essential components of the equipment.

For ion exchange chromatography a suppressor column is installed between main column and detector. The mobile phase is supplied from one or several reservoirs and flows through the column, usually at a constant rate, and then through the detector. Any part of the system that is in contact with the mobile phase should be constructed of materials inert to corrosive components of the mobile phase. The entire system dead volume has to be kept at the minimum. The tubing length and diameter of plumbing between the injector, column and detector has to be kept at the minimum. Higher volumes in these connections lead to increased dispersion and tailing of peaks.

Pumping systems

The pumping systems deliver metered amounts of the mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient elution chromatography, or to make an isocratic mobile phase (i.e., mobile phases having a fixed ratio of solvents). The system should be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time. Pumps may be provided with a mechanism for 'bleeding' the system of any entrapped air.

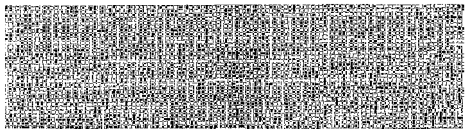
Injectors

After dissolution in the mobile phase or other suitable solvent, samples that are to be chromatographed are injected, either manually by a syringe or by fixed-loop injectors, or automatically by autosamplers. An autosampler consists of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer samples from the vials to a loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables like temperature. Manual partial filling of loops is not recommended because of poorer injection volume precision.

Stationary phases and columns

The different types of stationary phases employed in liquid chromatography are:

- silica, alumina or porous graphite, used in normal-phase chromatography, wherein separation is based on the principle of adsorption and/or mass distribution,
- chemically modified silica, polymers or porous graphite, used in reversed-phase chromatography, wherein separation is based principally on partition of the molecules between the mobile phase and the stationary phase,



- resins or polymers with acid or basic groups, used in ion exchange chromatography, wherein separation is based on competition of the ions to be separated with those in the mobile phase,
- porous silica or polymers, used in size-exclusion chromatography, wherein separation is based on differences between the volumes of the molecules to be separated and the corresponding steric exclusion,
- stationary phases like cellulose or amylose derivatives, proteins or peptides, cyclodextrins, etc., chemically modified specially for chiral chromatography for separation of enantiomers.

For most pharmaceutical analysis, separation is achieved by partitioning of compounds in the test solution between the mobile and stationary phases. Systems consisting of polar stationary phases and non-polar mobile phases are described as normal phases while the opposite arrangement (i.e., polar mobile phases and non-polar stationary phases) are called reversed-phases. In reversed-phase chromatography utilising chemically modified silica, the silanol groups of silica on the surface of the support react with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase influences the separation properties of the chromatographic system. Commonly used bonded phases are octyl (C_8), octadecyl (C_{18}), phenyl (C_6H_5), cyanopropyl (CN), aminopropyl (NH_2) and diol.

Unless otherwise stated by the manufacturer, silica-based reversed-phase columns are considered to be stable in a mobile phase having an apparent pH in the range 2.0 to 8.0. It is advisable to use a pre-column before the analytical column while using mobile phases of high pH with a silica-based column. Columns composed of porous graphite or particles of polymeric materials like styrene-divinylbenzene copolymer are stable over a wider range of pH. Making the mobile phase more or less polar controls the affinity of a compound for the stationary phase, and thus its retention time on the column. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

For analytical separations, the particle size of the most commonly used stationary phases varies between 3 µm and 10 µm. The particles may be spherical or irregular, of varying porosity and specific surface area. These parameters contribute to the chromatographic behaviour of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g., expressed as the carbon loading, and whether the stationary phase is end capped (i.e. residual silanol groups are silylated) or base deactivated are additional determining factors. When residual silanol groups are present, tailing of peaks particularly of basic substances can occur.

Base deactivation of the stationary phases is carried out by removal of basic components by careful washing and hydrolysing most of the superficial siloxane bridges.

Columns are usually made of stainless steel unless otherwise specified in the monograph. For analytical chromatography, they may be of varying length and internal diameter. Columns with internal diameters of less than 2 mm are often referred to as microbore columns.

In ion exchange chromatography, the column is made of a rigid material, usually plastic. It is generally 5 cm to 30 cm long with an internal diameter of 2 mm to 10 mm. The stationary phase is usually in the form of small diameter particles, 5 µm to 10 µm, uniformly packed into the column. The cation exchangers are usually obtained by sulphonation and anion exchangers by attaching quaternary ammonium groups to the polymeric resins.

The temperature of the mobile phase and the column must be kept constant during an analysis. Most separations are performed at ambient conditions, but columns may be heated to give higher efficiency. In order to reduce the possibility of degradation of the stationary phase or of occurrence of changes in the composition of the mobile phase, heating of the columns above 60° is not recommended. In some cases a particular commercial brand of column that has been found suitable is mentioned, but such statements do not imply that a different but equivalent commercial brand cannot be used. It may be necessary with a particular chromatograph to modify the conditions specified in the monograph but it should be ensured that comparable results are obtained under the modified conditions.

Detectors

A detector consists of a flow through cell mounted at the end of the column and capable of detecting various types of components in the eluate. The recommended volume of the detector flow cell is 3 µl to 20 µl. Ultraviolet/visible (UV/Vis) spectrophotometers, including diode array detectors, are the most commonly employed detectors. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. The wavelength setting is specified in the individual monograph.

Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used. Fluorimetric detectors are sensitive to compounds that are fluorescent or that can be converted to fluorescent derivatives. Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing

chromatographed compound as it emerges from the column. Electrochemical detectors are suitable for measuring nanogram quantities of easily oxidisable compounds like phenols and catechols.

Conductivity detector is the detector of choice in ion exchange chromatography. UV-visible detectors for absorbing species, indirect UV-visible detectors, amperometric detectors and fluorescence detectors are also employed for specific applications.

Mobile phases

In case of normal-phase chromatography, less polar solvents (*e.g. hexane, dichloromethane*) are employed. The presence of water or polar solvents in the mobile phase is to be strictly controlled to obtain reproducible results. In reversed-phase chromatography, aqueous mobile phases or polar solvents with or without organic modifiers are employed. Components of the mobile phase are usually filtered to remove particles greater than 0.45 µm. Multicomponent mobile phases are prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by mixing. Alternatively, individual pumps controlled by proportioning valves, which mix the solvents in the desired proportion, may deliver the solvents. It is advisable to have the mobile phase solvents or solvent mixtures degassed using a vacuum pump or other suitable means that will not affect the composition of the mixture. For accurate quantitative analysis, high purity reagents and HPLC grade organic solvents must be used. Adjustment of the pH, if necessary, is effected using the aqueous component of the mobile phase. The system is flushed with a mixture of water and the organic modifier of the mobile phase (in a suitable composition) after the completion of chromatography when buffer solutions are used. On completion of the analysis, it is necessary to wash the column with appropriate solvent followed by storage in recommended solvent. During storage, both the ends of column need to be plugged properly to prevent drying of the column bed.

A counter-ion for ion-pair chromatography or a chiral selector for chromatography using an achiral stationary phase may also be used to modify mobile phases.

Data acquisition systems

Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas as well as sample identification and method variables. Data may be collected on simple recorders for manual measurement or on stand alone integrators, which range in complexity from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

Method

Equilibrate the column with the prescribed mobile phase and flow rate, at room temperature or at a temperature specified in the monograph, until a stable baseline is achieved. Prepare the test and standard solutions as described in the individual monograph. The solutions should be free from solid particles. A blank injection of the mobile phase and the sample diluent should be carried out and monitored during the test to detect any interference.

Determinations

General

Normalisation procedure — The percentage content of one or more components of the substance under examination is calculated by determining the area of the peak or peaks as a percentage of the total area of all the peaks, excluding those due to solvents or any added reagents and those below the 'ignore' limit.

Internal standard procedure — Equal amounts of a component that gets resolved from the substance under examination (*the internal standard*) are added to the test solution and a reference solution. The internal standard shall be one that does not react with the substance under examination, shall be stable and shall not contain impurities with a retention time similar to that of the substance under examination. The concentration of the substance under examination is determined by comparing the ratio of the peak areas or peak heights due to the substance under examination and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the reference substance and the internal standard in the reference solution.

External standard procedure — The concentration of the component(s) to be analysed is determined by comparing the response(s) obtained with the test solution to the response(s) obtained with a reference solution.

Response factor — The sensitivity of a detector is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The sensitivity of a detector relative to a standard substance is expressed as response factor.

Relative response factor (RRF) — Relative response factor of an impurity is the ratio of the peak response of the impurity to that of an equal amount of the drug substance in the conditions prescribed in the test.

Correction factor – This is the reciprocal of the relative response factor (RRF) and if given in related substances test of individual monograph, area of impurity peak shall be multiplied with correction factor.

Secondary peaks — A secondary peak is a peak in the chromatogram other than the principal peak and any peaks

due to internal standard, solvent or derivatising agents.Peaks identified as due to the counter-ion and/or other excipients including preservatives in the substance under examination may also be excluded.

Quantitative methods

For assays and for quantitative estimations of components, the internal standard procedure or the external standard procedure may be adopted. The normalisation procedure is not normally applied for such determinations. In tests for related substances, either the external standard procedure with a single reference solution or the normalisation procedure may be used. However, with both these methods, when a dilution of the test solution is used for comparison, the responses of the related substances should be similar to the substance itself (response factor of 0.8 to 1.2); otherwise correction factors may have to be applied.

Peak areas and peak heights are usually proportional to the quantity of compounds eluting. Peak areas are generally used but may be less accurate if peak interference occurs. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided.

Related substances tests are sometimes based on the determination of peaks due to impurities, expressed as a percentage of the area due to the drug peak. However, it is preferable to compare impurity peaks to the chromatogram of a standard at a similar concentration. The standard may be the drug itself at a level corresponding to say, 0.5 per cent impurity, or in the case of toxic or signal impurities, a standard of the impurity itself.

When the test for related substances prescribes the summation of impurities or there is a quantitative determination of an impurity, an appropriate threshold setting and appropriate conditions for the integration of the peak areas should be chosen. In such tests the 'ignore' limit, e.g. the areas of peaks whose areas are below the limit are not taken into account, is generally 0.05 per cent. Thus, the threshold setting of the data collection system corresponds to, at least, half of the ignore limit.

Peaks due to the solvent(s) used to dissolve the sample are also to be ignored.

System suitability

This is an integral part of liquid chromatographic method for assuring adequate performance of the system. Because of normal variations in equipment, supplies and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable. System suitability also verifies that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.

Replicate injections of a standard solution used in the assay or other standard solution are compared to determine whether the requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0 per cent or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0 per cent.

The following requirements and any supplementary requirements given in the individual monograph are to be fulfilled unless otherwise prescribed:

- In a related substances test or assay, for a peak in the chromatogram obtained with a reference solution used for quantification, the symmetry factor is 0.8 to 1.5, unless otherwise prescribed,
- In an assay of an active substance, where the value is 100 per cent for a pure substance, the maximum permitted relative standard deviation $s_r(\text{per cent})_{max}$ for the defined limits is calculated for a series of injections of the reference solution using the following equation:

$$s_r\,(per\ cent)_{\max} = \frac{KB\sqrt{n}}{t_{90\ per\ cent,\,n-1}}$$

where, K = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\ per\ cent,\,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required per cent relative standard deviation after six injections for B = 1.0,

B = upper limit given in the definition of the individual monograph minus 100 per cent,

n = number of replicate injections of the reference solution ($3 \le n \le 6$),

$t_{90\ per\ cent,\,n-1}$ = Student's t at the 90 per cent probability level (double sided) with n-1 degrees of freedom.

	Number of Individual Injections			
	3	4	5	6
B (per cent)	Maximum Permitted Relative Standard Deviation			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

Unless otherwise prescribed, the maximum permitted relative standard deviation does not exceed the appropriate value given in table 1 of repeatability requirements. This requirement does not apply to the tests for related substances.

Table 1. Repeatability requirements

–	In a related substances test, the limit of quantification (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. Depending on various factors, such as the frequency of use of the procedure and experience with the chromatographic system, the analyst chooses an appropriate verification scheme to monitor this.

Replicate injections of the standard solution may be made before the injection of samples or may be interspersed among sample injections. System suitability must be demonstrated throughout the run by injection of an appropriate control preparation at appropriate intervals, including at the end of the analysis. The control preparation can be the standard solution used in the test or a solution containing a known amount of analyte.

No sample analysis is acceptable unless the requirements of system suitability have been met. Test results obtained while the system fails system suitability requirements are unacceptable.

Adjustment of chromatographic conditions

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below. Adjustment of conditions with gradient elutions is more critical than with isocratic elutions, since it may lead to shifts in peaks to a different step of the gradient, thus leading to the incorrect assignment of peaks, and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time. Changes other than those indicated require revalidation of the method. The chromatographic conditions described have been validated during the elaboration of the monograph.

The system suitability tests are included to verify that the separation required for satisfactory performance of the test or assay is achieved. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reversed-phase liquid chromatographic methods in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel), which exhibits the desired chromatographic behaviour.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Isocratic elution

Composition of the mobile phase. The amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; no other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase. ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase. ± 10 per cent.

Flow rate. ± 50 per cent; a larger adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters

Stationary phase

- no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C_{18} by C_8),
- *particle size*: maximum reduction of 50 per cent; no increase permitted.

Column dimension

- *length*: ± 70 per cent,
- *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 \, d_2^2}{l_1 \, d_2^2}$$

where, F_1 = flow rate indicated in the monograph, in millilitres per minute,

F_2 = adjusted flow rate, in millilitres per minute,

l_1 = length of the column indicated in the monograph, in millimetres,

l_2 = length of the column used, in millimetres,

d_1 = internal diameter of the column indicated in the monograph, in millimetres,

d_2 = internal diameter of the column used, in millimetres.

Temperature. ± 10°, where the operating temperature is specified, unless otherwise prescribed.

Detector wavelength. No adjustment permitted.

Injection volume. May be adjusted as far as it is consistent with accepted precision, linearity, and detection limits. Note that excessive injection volume can lead to unacceptable band broadening, causing a reduction in column efficiency and resolution.

Gradient elution

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

Composition of the mobile phase/gradient elution. Minor adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- the system suitability requirements are fulfilled,
- the principal peak(s) elute(s) within ± 15 per cent of the indicated retention time(s),
- the final composition of the mobile phase is not weaker in elution power than the prescribed composition.

Where compliance with the system suitability requirements cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

Dwell volume. The configuration of the equipment employed may significantly alter the resolution, the retention time and the relative retentions described. Should this occur, it may be due to excessive dwell volume. Monographs preferably include an isocratic step before the start of the gradient programme so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the systems used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points (t min) stated in the gradient table may be replaced by adapted time points (t_C min), calculated using the following equation.

$$t_C = t - \frac{(D - D_O)}{F}$$

where, D = dwell volume, in millilitres,
D_O = dwell volume used for development of the method, in millilitres,
F = flow rate, in millilitres per minute.

The isocratic step introduced for this purpose may be omitted if validation data for application of the method without this step is available.

pH of the aqueous component of the mobile phase. No adjustment permitted.

Concentration of salts in the buffer component of a mobile phase. No adjustment permitted.

Flow rate. Adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters

Stationary phase

- no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C_{18} by C_8),
- *particle size*: no adjustment permitted.

Column dimensions

- *length*: ± 70 per cent,
- *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 \, d_2^2}{l_1 \, d_2^2}$$

Where, F_1 = flow rate indicated in the monograph, in millilitres per minute,
F_2 = adjusted flow rate, in millilitres per minute,
l_1 = length of the column indicated in the monograph, in millimetres,
l_2 = length of the column used, in millimetres,
d_1 = internal diameter of the column indicated in the monograph, in millimetres,
d_2 = internal diameter of the column used, in millimetres.

Temperature. ± 5°, where the operating temperature is specified, unless otherwise prescribed.

Detector wavelength. No adjustment permitted.

Injection volume. May be adjusted as far as it is consistent with accepted precision, linearity, and detection limits. Note that excessive injection volume can lead to unacceptable band broadening, causing a reduction in column efficiency and resolution.

Fig. 2.4.14-1 is a graphical representation of the common events during chromatography and assists in understanding the various terms more commonly employed and discussed below.

Resolution

The resolution or resolution factor, R, is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. Resolution between peaks of similar height of two components may be defined by the expression:

$$R = \frac{2 (V_{Rb} - V_{Ra})}{W_2 + W_1}$$

Where, V_{Rb} and V_{Ra} = retention times or distances along the baseline between the point of

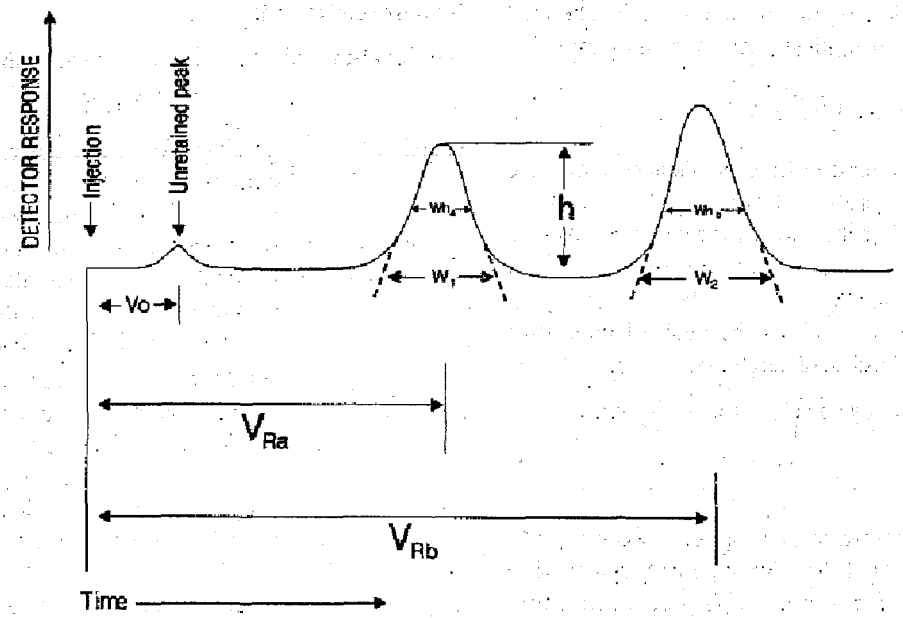


Fig. 2.4.14-1

injection and perpendiculars dropped from the maxima of two adjacent peaks,

W_2 and W_1 = corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

Where electronic integrators are used, it may be convenient to determine the resolution, R, by the equation

$$R = \frac{1.18\,(V_{Rb} - V_{Ra})}{W_{ha} + W_{hb}}$$

Where, V_{Rb} and V_{Ra} = retention times or distances along the baseline between the point of injection and perpendiculars dropped from the maxima of two adjacent peaks,

W_{hb} and W_{ha} = the respective peak widths measured at half-peak height.

The values of W_{ha}, W_{hb}, V_{Ra} and V_{Rb} must be expressed in the same unit of measurement.

Peak-to-valley ratio

The peak-to-valley ratio (p/v) may be used as a system suitability requirement in a test for related substances when baseline separation between two peaks is not reached.

$$p/v = \frac{H_p}{H_v}$$

where, H_p = height above the extrapolated baseline of the minor peak,

H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

Capacity factor

The capacity factor, also called mass distribution ratio, K', is stated in the monograph. It is defined by the expression

$$K' = (V_{Rb} - V_o)/V_o$$

where, V_{Rb} = retention time or distance along the baseline between the point of injection and perpendicular dropped from the maximum of the peak of interest,

V_o = the distance along the baseline between the point of injection and perpendicular dropped from the maximum of the peak of an unretained component.

The values of V_{Rb} and V_o must be expressed in the same unit of measurement.

Column efficiency

Column efficiency can also be used as a system suitability requirement. It is a measure of peak sharpness, which is

important for the detection of trace components. It is defined in terms of the number of theoretical plates, N, by the expression

$$N = 5.54\ V_R^{\ 2} / W_h^{\ 2}$$

Where, V_R = retention time or distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the peak of interest,

W_h = the width of the peak of interest at half-peak height.

The values of V_R and W_h must be expressed in the same unit of measurement.

Symmetry factor

Symmetry factor or tailing factor, S, of a peak (Fig. 2.4.14-2) is a measure of peak symmetry. It is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. It is calculated from the expression

$$S = W_x / 2A$$

where, W_x = the width of the peak at 5.0 per cent of the peak height,

A = the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5.0 per cent of the peak height.

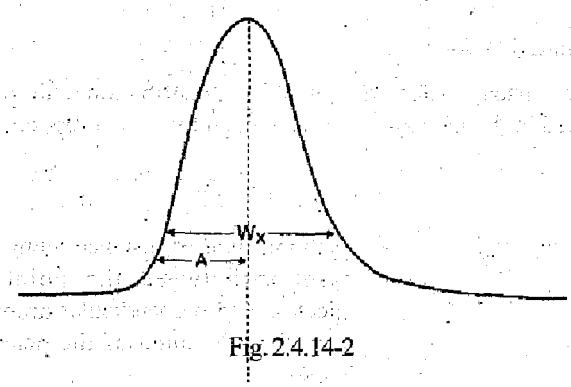


Fig. 2.4.14-2

Relative retention

Relative retention, $r_{a/b}$, is calculated as an estimate from the expression

$$r_{a/b} = \frac{t_{r,b}}{t_{r,a}}$$

where, $t_{r,b}$ = retention time of the peak of interest,

$t_{r,a}$ = retention time of the reference peak (usually the peak corresponding to the substance under examination).

Signal-to-noise ratio

The signal-to- noise ratio is determined from the expression:

$$S/N = 2H / h$$

where, H = height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height,

h = range of the background noise in a chromatogram obtained after injection of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

2.4.15. Paper Chromatography

Paper chromatography is a technique in which separation of the components of a mixture is achieved through the action of a single liquid phase in a process similar to adsorption chromatography in columns and in which a sheet of paper of suitable texture and thickness acts as the adsorbant. Since the natural water content of the paper, or selective inhibition of a hydrophilic component of the liquid phase by the paper may be regarded as a stationary phase, a partitioning of the solute between two phases may contribute to the separation of components.

Alternatively, a two-phase system may be adopted in which the paper is impregnated with one of the phases, which then remains stationary and the chromatogram is developed by slow movement of the other, mobile phase over the sheet. Development may be ascending in which case the mobile phase is carried up the paper by capillary forces, or descending in which case the mobile phase is also assisted by gravitational force.

Apparatus

(a) A vapour-tight tank of glass, porcelain or stainless steel provided with inlets for addition of solvent or for releasing internal pressure and so designed that the progress of the chromatographic run can be observed without opening the tank.

(b) A rack of corrosion-resistant material about 5 cm shorter than the inside height of the tank, to serve as support for the solvent trough and for antisiphoning rods which in turn, hold the chromatographic sheets.

(c) Glass troughs, longer than the width of the chromatograph sheets and holding a volume of solvent greater than that needed for one chromatographic run.

(d) Antisiphoning rods of heavy glass to be supported by the rack, and running outside of, parallel to and slightly above the edge of the glass trough.

(e) Chromatographic sheets of special filter paper not less than 25 mm wide and not wider than the length of the troughs, cut into strips to a length approximately equal to the height of the tank; the paper is cut so that the mobile phase runs in the direction of the grain of the paper. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that, when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few cm below the rods. Care should be taken to avoid contaminating the filter paper by excessive handling or by contact with dusty surfaces.

Descending Paper Chromatography

Method. The substance under examination is dissolved in a suitable solvent. The volumes of the resulting solution are applied by means of micropipette in 6 to 10 mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added, or alternatively, the solution may be applied in the form of bands (10 to 20 mm x 2 to 6 mm unless otherwise specified) along the pencil line.

Use sufficient of the saturating solvent prescribed in the monograph to form a 25 mm layer in the bottom of the tank. Close the tank and allow to stand for 24 hours at room temperature.

Suspend the spotted chromatographic sheet in the tank by the use of antisiphoning rods which hold the upper end of the sheet in the solvent trough. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the tank without touching the rack or the tank walls or the fluid in the tank.

Close the tank and allow the paper to stand in it for 1.5 hours. Introduce through the inlet into the solvent trough a sufficient quantity of the prescribed mobile phase, close the tank and allow development to proceed for the distance or the time prescribed in the monograph, protecting the paper from bright light during development. Remove the paper from the tank and allow it to dry in air at the temperature specified in the monograph. Visualise the spots as described in the monograph.

The paper section(s) (see under Apparatus) predetermined to contain the separated components may be cut out and eluted by an appropriate solvent, and the solutions may be made up to a known volume and quantitatively analysed by appropriate chemical or instrumental methods.

Ascending Paper Chromatography

Method. The test materials are applied to the chromatographic sheets as directed under Descending Paper Chromatography and above the level to which the paper is dipped into the developing solvent. The top of the tank contains a device from which the paper is suspended, and which is capable of being lowered without opening the tank. In the bottom of the tank is a trough to contain the mobile phase into which the paper may be lowered.

Use sufficient of the prescribed mobile phase to form a 25 mm layer in the trough. If a two-phase system is used, both phases are added. If prescribed, pour the saturating solvent between the trough and the walls of the tank. Close the tank and allow to stand for 24 hours at room temperature. Insert the prepared paper into the tank, close the lid and allow to stand for 1½ hours. Lower the paper into the mobile phase and allow development to proceed for the distance or the time prescribed in the monograph, protecting the paper from bright light during development. Remove the paper from the tank and allow it to dry in air at the temperature specified in the monograph. Qualitative and quantitative analyses of the spots may be conducted as described under Descending Paper Chromatography.

Adjustment of chromatographic conditions

Composition of the mobile phase. The amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; for a minor component at 10 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 7-13 per cent whereas a 2 per cent absolute adjustment allows a range of 8-12 per cent, the relative value therefore being the larger; for a minor component at 5 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 3.5-6.5 per cent whereas a 2 per cent absolute adjustment allows a range of 3-7 per cent, the absolute value being the larger in this case; no other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase. ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase. ± 10 per cent.

Application volume. 10-20 per cent of the prescribed volume if using fine particle size plates (2-10 µm).

2.4.16. Size-Exclusion Chromatography

Size-exclusion chromatography is a technique of separation of molecules in solution according to their size. It is based on the repeated exchange of solute molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the column-packing material. The pore-size range of the packing material determines the molecular-size range within which separation can take place.

Molecules small enough to penetrate all the pore spaces elute at the total permeation volume (V_t). Molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the exclusion volume (V_O void volume). Separation according to molecular size takes place between the exclusion volume and the total permeation volume with useful separation occurring in the first two thirds of this range.

Apparatus

A chromatographic column, temperature-controlled, if necessary, packed with a separation material capable of fractionation in the appropriate range of molecular sizes and through which the eluent is passed at a constant rate. The dimensions of the column are stated in the individual monograph as (length x internal diameter). The mobile phase is passed through the column either by gravity or by means of a pump. The outlet from the column is connected to a detector fitted with an automatic reorder that allows the monitoring of the relative concentrations of the components of the sample. Detectors are usually based on photometric, refractometric or luminescent properties. An automatic fraction-collector may be attached, if required.

The packing material may be a soft support such as a swollen gel or a rigid support such as glass, silica or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurised systems giving faster separations. Before carrying out the separation, the packing material is treated, and the column is packed as described in the monograph, or according to the manufacturer's instructions.

The temperature of the column, if other than that of the room, the nature of the packing material, the composition and flow rate of the mobile phase and the means of detection are stated in the individual monograph.

Performance

The column efficiency may be derived as described under Gas chromatography (2.4.13) but the term in the expression for calculation is called the retention volume (V_R) for the component of interest. The retention volume is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the peak of interest.

The distribution coefficient (K_D), where stated in the monograph, is calculated from the expression

$$K_D = \frac{(V_R - V_O)}{(V_T - V_O)}$$

The values of V_R, V_O, and V_T must be expressed in the same unit of measurement.

Determination of relative component composition

If all of the components of the sample under examination exhibit equivalent responses to the detector, then the relative amount of each component can be determined by dividing each peak area by the sum of the peak areas of the components of interest. If the responses are not equivalent, calculate the relative component composition either from the calibration curves obtained with the calibration standards specified in the monograph or by any other means.

Determination of molecular weight

Carry out the method on the substance under examination and calibration standards using the procedure given in the individual monograph. Plot a graph of the retention volume of the standards as a function of the logarithm of the molecular weight. The curve is almost a straight line within the exclusion and total permeation limits. The molecular weight of the component of interest may be estimated from the calibration curve. The calibration is valid only for the particular system used under the specified experimental conditions.

Materials

Agarose FC. For the separation of proteins with molecular weights of 6×10^4 to 2×10^7 and of polysaccharides with molecular weights of 3×10^3 to 5×10^6. They occur as swollen beads 60 to 140 µm in diameter and are available as a 4 per cent suspension in *water*.

Agarose FC, Cross-linked. It is prepared from agarose by reaction with 2,3-dibromo-1-propanol in strongly alkaline conditions. It is used for separation of proteins with molecular weights of 6×10^4 to 2×10^6 and of polysaccharides of the same range of molecular weights as with Agarose FC.

Silica Gel FC. For the separation of proteins with molecular weights of 1×10^3 to 3×10^5. It occurs as a very finely divided powder with an average particle size of about 10 µm with a very hydrophilic surface and an average pore diameter of about 30 nm. It is compatible with aqueous solutions of pH 2 to 8 and with organic solvents.

2.4.17. Thin-Layer Chromatography

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent.

Identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation.

Apparatus

(a) Flat glass plates of appropriate dimensions which allow the application at specified points of the necessary quantities of the solution being examined and appropriate reference solutions and which allow accommodation of the specified migration path-length. The plates are prepared as described below; alternatively, commercially available pre-coated plates may be used.

(b) An aligning tray or a flat surface on which the plates can be aligned and rested when the coating substance is applied.

(c) The adsorbent or coating substance consisting of finely divided adsorbent materials, normally 5 µm to 40 µm in diameter, suitable for chromatography. It can be applied directly to the plate or can be bonded to the plate by means of Plaster of Paris (Hydrated Calcium Sulphate) or with any other suitable binders. The adsorbent may contain fluorescing material to help in visualising spots that absorb ultraviolet light.

(d) A spreader which, when moved over the glass plate, will apply a uniform layer of adsorbent of desired thickness over the entire surface of the plate.

(e) A storage rack to support the plates during drying and transportation.

(f) A developing chamber that can accommodate one or more plates and can be properly closed and sealed. The chamber is fitted with a plate support rack that supports the plates, back to back, with the lid of the chamber in place.

(g) Graduated micro-pipettes capable of delivering microlitre quantities say 10 ml and less.

(h) A reagent sprayer that will emit a fine spray and will not itself be attacked by the reagent.

(i) An ultraviolet light, suitable for observation at short (254 nm) and long (365 nm) ultraviolet wavelengths.

Preparation of plates. Unless otherwise specified in the monograph, the plates are prepared in the following manner. Prepare a suspension of the coating substance in accordance with the instructions of the supplier and using the spreading device designed for the purpose, spread a uniform layer of the suspension, 0.25 to 0.30 mm thick, on a flat glass plate 20 cm long. Allow the coated plates to dry in air, heat at 100° to 105° for at least 1 hour (except in the case of plates prepared with cellulose when heating for 10 minutes is normally sufficient) and allow to cool, protected from moisture. Store the plates protected from moisture and use within 3 days of preparation. At the time of use, dry the plates again, if necessary, as prescribed in the monograph.

Method

Unless unsaturated conditions are prescribed, prepare the tank by lining the walls with sheets of filter paper; pour into the tank, saturating the filter paper in the process, sufficient of the mobile phase to form a layer of solvent 5 to 10 mm deep, close the tank and allow to stand for 1 hour at room temperature. Remove a narrow strip of the coating substance, about 5 mm wide, from the vertical sides of the plate. Apply the solutions being examined in the form of circular spots about 2 to 6 mm in diameter, or in the form of bands (10 to 20 mm x 2 to 6 mm unless otherwise specified) on a line parallel with, and 20 mm from, one end of the plate, and not nearer than 20 mm to the sides; the spots should be 15 mm apart. If necessary, the solutions may be applied in portions, drying between applications. Mark the sides of the plate 15 cm, or the distance specified in the monograph, from the starting line. Allow the solvent to evaporate and place the plate in the tank, ensuring that it is as nearly vertical as possible and that the spots or bands are above the level of the mobile phase. Close the tank and allow to stand at room temperature, until the mobile phase has ascended to the marked line. Remove the plate and dry it.

For two-dimensional chromatography dry the plate after the first development and carry out the second development in a direction perpendicular to the first.

When the method prescribed in the monograph specifies 'protected from light' or 'in subdued light' it is intended that the entire procedure is carried out under these conditions.

Adjustment of chromatographic conditions

Minor adjustments to the parameters of the test may be made in order to satisfy the system suitability criteria. These may be:

Mobile phase. Minor solvent component of a mixture: ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; no other component altered by more than 10 per cent absolute;

Concentration of salts. In the buffer component of the mobile phase; ± 10 per cent;

pH of the aqueous component of the mobile phase. ± 0.2 pH, unless otherwise stated in the monograph, or ± 1.0 pH, when non-ionisable substances are to be examined;

Volume of solutions applied. 10-20 per cent of the prescribed volume if plates have fine particles (2-10 µm).

Visualisation

After development, the plate should be examined under an ultraviolet light having a maximum output at about 254 nm or at about 365 nm, as the case may be. Alternatively, it may be visualised as directed in the monograph; where a spraying technique is prescribed it is essential that the reagent be evenly applied as a fine spray.

The term secondary spot means any spot other than the principal spot. Similarly, a secondary band is any band other than the principal band.

Semi-quantitative estimation

Identification. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution in respect of the colour, the size and the Rf of the spots.

Test for Related substances. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity (ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Secondary spots. A secondary spot is a spot in the chromatogram other than the principal spot and any spots due to internal standard, solvent or derivatisting agents. If justified, spots identified as due to the counter-ion and/or other excipients including preservatives in the substance under examination may also be excluded.

Quantitative measurement

The substances that have been separated after development of the plate and that respond to UV-Vis irradiation can be estimated directly on the plate with suitable instrumentation. Measurement is of the reflectance of the incident light from the spots by moving the plate or the measuring device. Likewise, fluorescence may be measured using an appropriate optical system.

Apparatus. The apparatus for direct measurement consist of:

- a device for exact positioning and reproducible application of the amount of solutions onto the plate,
- a mechanical device for moving the plate or the measuring device along the *x*-axis or the *y*-axis,
- a recorder and a suitable integrator or a computer, and
- a photometer with a source of light, an optical device for generating monochromatic light and a photo cell of adequate sensitivity; for measurement of fluorescence, a suitable filter to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass.

Method. Prepare the test solution and reference solutions as prescribed in the individual monograph. Use the same solvent for all the solutions and apply the same volume of each and develop the plate. Prepare and apply not fewer than 3 reference solutions of the substance under examination, the concentrations of which span the expected value in the test solution (about 80 per cent, 100 per cent and 120 per cent). Treat with the prescribed reagent, if necessary, and record the reflectance, the transmittance or fluorescence in the chromatograms obtained with all the solutions. Use the measured results to calculate the amount of substance in the test solution.

The requirement for resolution and separation are prescribed in the individual monograph.

Procedure for Bacitracin, Neomycin and Polymyxin B

The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage forms when present singly and in two- and three-component mixtures. Prepare a test solution as follows, unless otherwise directed in the individual monograph.

Test Solution

For Drug Substances. Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulphate, or Polymyxin B Sulphate in *0.1 M hydrochloric acid* to obtain a solution containing about 500 Bacitracin Units per ml, 3.5 mg of neomycin (base) per ml, or 10,000 Polymyxin B Units per ml.

For Solutions. Where the Solution contains neomycin and polymyxin B, dilute a portion of it with *0.1 M hydrochloric acid* to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per ml. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with *0.1 M hydrochloric acid* to obtain a solution containing about 10,000 Polymyxin B Units per ml.

For Creams, Lotions and Ointments. Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc,

transfer a portion of it equivalent to about 500 Bacitracin Units, to a 15-ml centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per ml to a 15-ml centrifuge tube. Add 4 ml of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 ml of *0.1 M hydrochloric acid*, vortex for 4 minutes, centrifuge, and use the clear supernatant.

Standard Bacitracin Solution. Dissolve a portion of *bacitracin zinc IPRS* in *0.1 M hydrochloric acid* to obtain a solution containing 500 Bacitracin Units per ml.

Standard Neomycin Solution. Dissolve a portion of *neomycin sulphate IPRS* in *0.1 M hydrochloric acid* to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per ml.

Standard Polymyxin B Solution. Dissolve a portion of *Polymyxin B Sulphate IPRS* in *0.1 M hydrochloric acid* to obtain a solution containing 10,000 Polymyxin B Units per ml. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of *Polymyxin B Sulphate IPRS* in *0.1 M hydrochloric acid* to obtain a solution containing 500*J* Polymyxin B Units per ml, *J* being the ratio of the labeled amount of Polymyxin B Units to the labeled amount of Bacitracin Units in each g of Cream, Lotion, or Ointment.

Developing Solvent Solution. A mixture of 4 volumes of *methanol*, 2 volumes of *isopropyl alcohol*, 2 volumes of *methylene chloride*, 2 volumes of *ammonium hydroxide*, and 1.5 volumes of *water*.

Procedure. Apply 10 µl of the test solution and each of the relevant standard solutions to a suitable thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber, and develop the chromatogram with the Developing Solvent System until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105° for 10 minutes. Spray the plate with a 0.2 per cent olution of *ninhydrin* in *butyl alcohol*, and heat at 105° for 5 minutes. The R_f value of each principal spot in the chromatogram of the test solution corresponds to that of the principal spot in the chromatogram obtained from each relevant standard solution as appropriate for the labeled active ingredient or ingredients specified on the label. If the chromatogram of the test solution yields excessive streaking, proceed as directed for Modified Procedure.

Modified Procedure. Transfer the test solution to a 15-ml centrifuge tube, add 10 ml of *saturated aqueous picric acid solution (1.2 per cent, w/v)*, vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-ml portions of *water* until no yellow color is observed in the washing. Discard the washings, and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 ml of *acetone*, add 1 ml of a freshly prepared solution of *sulphuric acid* in *acetone* (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 ml of *acetone*, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 ml of *0.1 M hydrochloric acid*. Repeat the procedure using this Modified Test Solution instead of the test solution. The R_f value of each principal spot in the chromatogram of the Modified Test Solution corresponds to that of the principal spot in the chromatogram obtained from each relevant Standard Solution as appropriate for the active ingredient or ingredients specified on the label.

Adoption of HPTLC for Herbs and Herbal Products

High-performance thin-layer chromatography (HPTLC) is the advanced and standardized version of TLC (see 2.4.17. Thin Layer Chromatography). HPTLC is actively applied in qualitative and quantitative analysis of a wide range of articles, including herbs and herbal products. It is known for its high separation power, performance, reproducibility, simplicity and is considered modern replacement to classical TLC. Its main use is to identify herbal and their extracts by their broad "chemical composition fingerprint using HPTLC" from electronic images of chromatograms, detect adulterants and secondly, using scanning densitometryto quantify markers.

In HPTLC, the stationary phase consists of a uniform, typically 200-µm layer of porous (pore size 60 Å), irregular particles of silica gel with a size between 2 and 10 µm and an average particle size of 5 µm, plus a polymeric binder and a fluorescence indicator (F254) coated onto a support, which is typically a glass plate or aluminum foil. Other stationary phases, such as chemically bonded phases (C8, C18, CN, NH2, DIOL) or microcrystalline cellulose, are also available with and without a fluorescence indicator. Because of the greater separation efficiency of the fine particles in HPTLC, the chromatographic system is miniaturized, using smaller developing chambers and shorter developing distances of 6 - 8 cm, in comparison to classical TLC, where 12 - 15 cm is required for best separation. Therefore, less mobile phase and less time are required for chromatogram development. Another effect of miniaturization is the use of smaller sample volumes and the resulting possibility of analyzing more samples per plate than in TLC. In addition, improved layer quality due to smaller particle size positively affects the signal-to-noise ratio, and therefore improves the detectability of separated sample components.

Similar to other planar chromatography techniques, in HPTLC, the plate is an open system affected by environmental factors, which must be controlled carefully. In HPTLC, variables are controlled within narrow ranges using a rigorously standardized methodology and appropriate equipment, thus HPTLC enables

increased reproducibility of the chromatographic result from plate to plate.

For qualitative and semi quantitative analysis i.e. HPTLC fingerprinting for identification, a scanner is not essential. Scanner is required for quantification of markers. Each and every herbal sample is amenable to fingerprinting even if none of its constituents (markers) may be known or available.

HPTLC involves the following.

Handling of Plates

HPTLC plates are delicate and must be handled with care to avoid damaging the layer. When moving the plate, it should be touched only in the upper part above the region of chromatography. Labeling the plate should be with a soft pencil in the upper corner. The expected developing distance can be marked on the edge with a short pencil mark that goes down to the glass support. Marking the developing distance across the entire plate should be avoided. Aligning the front with a short mark on the edge is easier.

Plates should be stored in a place that is free of fumes and dust with the layer facing down. Shrink wrap of the package should not be in contact with the layer to avoid contamination with volatiles from the foil. Generally, HPTLC plates are ready for use without any pretreatment. Older or improperly stored plates may have accumulated impurities and therefore require precleaning. This is the case when after development, the solvent front (or additional secondary fronts) can be seen under UV 254 nm as an intense, broad dark band across the plate. Precleaning can be achieved by developing the plate in methanol to the upper edge. Subsequently, the plate is dried in a clean oven at 120° for 20 minutes. For cooling down to room temperature and for storage prior to further use, plates can be kept in an empty desiccator or wrapped in aluminum foil. Precleaning may slightly change the selectivity of a plate. Therefore, it is important to define in a method whether precleaned plates are to be used. Precleaning with the mobile phase is usually not a good option because it may be difficult to completely remove all components during the drying step.

Test solution

Sufficient amount of the sample is dissolved in a suitable solvent at typical concentrations of 0.1 - 1 mg per ml for individual substances. For herbs and herbal products, concentrations are 100 mg per ml in methanol or 70 per cent *ethanol*.

This is the sonicated for 15 minutes, centrifuges for 10 minutes and supernatant is used as test solution.

Sample under examination may also be prepared by processes such as infusion, decoction, maceration, sonication, soxhelation, centrifugation, percolation and other concentration techniques.

Reference solutions

A) Sufficient amount of the standard is dissolved, preferably, in the same solvent used for the Test solution and at a comparable concentration. For herbs and herbal products, selection of a suitable marker compound for a particular herb or herbal preparation for quantification is also of critical importance. The reference material used in the Standard solution should be an authenticated Indian Pharmacopoeia Botanical Reference Substance (IPBRS) and Indian Pharmacopoeia Reference Substance (IPRS). Unless otherwise stated in an individual monograph, IPRS is dissolved at a concentration of 1 mg per ml in *methanol*.

B) For essential oils fingerprint analysis, the IPRS and samples are dissolved in *toluene* at a concentration of 50 µl per ml.

C) For fixed oils, 25 µl of fatty oil IPRS as well as samples are dissolved in 3 ml of *dichloromethane* (*methylene chloride*) fingerprint analysis for identification.

Preparation of standard and sample solution: Dissolve about 20 mg of RS and samples in 3 ml *methylene chloride*.

Stationary phase

Selection of the stationary phase depends upon the nature of the compounds to be separated, however, 200 µm layers of irregular particles of HPTLC Si 60 F254 (silica gel with average particle size of 5 µm and pore size 60 Å and a fluorescence indicator F 254) is suitable for the majority of herbs and herbal products.

Mobile phase

The mobile phase should be freshly prepared. Selection of the mobile phase depends upon the nature of the compounds to be separated. The mobile phase may be a mixture of two or more solvents. A drop of acid or alkali may sharpen the bands. For identification of herbs and herbal products, a suitable mobile phase is one that creates a sequence of separated bands (fingerprint) well spread over the entire R_f range. For quantitative determination, target compounds should be between R_f 0.25 to 0.65 and well resolved from all other constituents of the samples. Migration of compounds generally correlates with their polarity. In normal phase separations, non-polar compounds migrate further and polar compounds migrate less far and *vice versa* in reverse phase chromatography.

Plate Layout and Sample Application

The standard format of the HPTLC plate is 20 × 10 cm (width × height). Other sizes (e.g., 10 × 10 cm) could be used as well, but one should keep in mind that for obtaining reproducible results, each size of plate requires a different chamber to maintain the same geometrical aspects as, for example, a twin-

trough chamber would have for the standard plate format. The optimum developing distance in HPTLC is 62 mm. Cutting plates to less than 10 cm in height provides no additional advantages.

Exact positioning is essential for proper identification of separated zones. Fig. 2.4.17-1 depicts the application of samples with respect to plate layout.

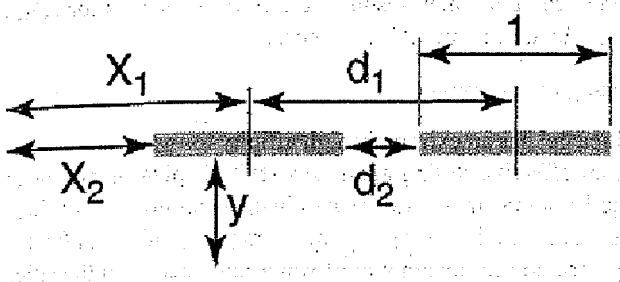


Fig. 2.4.17-1: Plate layout

For proper identification (migration distance/R_f values), all samples must be applied on a (virtual) line parallel to the lower edge of the plate. The distance (y) must be large enough to avoid the sample being immersed in the mobile phase. During development, the velocity of the mobile phase decreases with increasing developing distance. Consequently, the result depends on the application position relative to the level of the developing solvent. For reproducible results, the distance from the lower edge (y) must be kept constant, and the amount of developing solvent placed into the chamber must also be fixed. To minimize solvent consumption, twin-trough chambers are used. The level of developing solvent in such chambers is typically 5 mm. A meniscus is formed on the plate at the immersion line. A sample applied at y = 8 mm will be well above this meniscus. The distance of the application position from the left and right edges of the plate (x) must be enough to avoid the so-called "edge effect".

The position X_1 is typically called "application position". To avoid the edge effect, the length of the applied band (l) has to be taken into account when defining X_2 as the distance from the edge of the plate. A minimum of 15 mm is necessary for X_2. The distance between two samples (d_1) also considers the band length (l). For d_2, a minimum of 2-3 mm is recommended so that samples do not interfere with each other if larger volumes are applied. The band length (l) must be fixed for each method because it affects the concentration of samples per band if a defined volume of sample solution is applied. The applied quantity (across a defined band length) influences the intensity of the separated zones and may affect whether a particular zone of a fingerprint is visible. An argument for selecting shorter bands would be that more samples can be applied on the plate. However, because chromatogram evaluation is primarily performed visually, it must be noted that a band seems to look "sharper" (more like a band and less oblong) when it is >5 mm. A band length of 6-8 mm is a good compromise.

Sample application

Test and reference solutions can be applied by spraying the solutions without touching the plate (spray-on technique). During spray-on application, the sample's solvent is nebulized and evaporated, creating sharp zones regardless of the polarity of the solvent or the volume. Use of inert gas like nitrogen is recommended for nebulization since it also prevents the oxidation of the samples but air can also be used. Narrow homogeneous sample bands are applied using an automated applicator/sampler. This ensures high resolution.

Chromatogram development for

a) Herbals and Essential Oils

The processes in a chromatographic chamber are highly complex and difficult to describe. Unlike column chromatography, which is assumed to take place in equilibrated chromatographic systems, planar chromatography always begins in a state of nonequilibrium and never reaches equilibrium. The sample has been applied onto the plate, which becomes the stationary phase only when it comes in contact with the mobile phase in the chromatographic chamber. The advancing mobile phase must be able to dissolve the sample for chromatography to begin. Driven by capillary action, the mobile phase velocity decreases with increasing migration distance because the resistance of the stationary phase against the flow is also increasing. The optimum migration distance for mobile phases on HPTLC plates is about 6 cm. Depending on the viscosity of the mobile phase, development takes place between 10 and 20 minutes. To further move the solvent front just 10 mm will extend the developing time by 5 - 15 minutes, while moving an additional 20 mm will increase the developing time by 15 - 40 minutes. The contribution of the extra developing distance to improved separation is usually not justifiable, because the diffusion that occurs at decreased velocity of the mobile phase often offsets any gain in distance of separated zones.

Chamber saturation is established before placement of the plate into the chamber. The mobile phase is placed in both troughs of the chamber, and the filter paper in the rear trough is wetted thoroughly. Chamber saturation occurs once the liquid in the tank is in equilibrium with its own vapor, and this is a function of time. After 20 minutes, the chamber will generally achieve a reproducible degree of saturation. If the filter paper for saturation is to be re-used, it must be dried completely first. Different chambers may give different results, depending on the chamber's geometry, the presence or absence of liquid and filter paper in one of the troughs, and the waiting time before introducing the plate and so may be avoided.

The plate should be placed into the chamber so that it rests in a vertical position against the front wall while the layer faces the rear wall. The dry portion of the layer absorbs solvent vapor from the mobile phase in gas phase to achieve adsorptive saturation, which occurs when the surface is covered by a layer of solvent molecules. Generally, this process can lower R_f values and affect the selectivity of the separation. Depending on the degree of saturation, a part of the mobile phase may evaporate off the plate. Multicomponent mobile phases may be separated by the stationary phase because of different interactions of the constituents. This process is affected by the degree of preconditioning.

In an unsaturated chamber, the situation is different. The rear trough does not contain liquid or filter paper. The plate is introduced immediately after the solvent, which does not allow enough time to achieve gas–liquid saturation equilibrium. Preconditioning has little effect, while evaporation and possibly formation of secondary fronts dominate the chromatographic process. Both processes typically cause alterations in the chromatograms, thereby affecting the separation. Hence 20 minutes saturation is desirable.

HPTLC standard conditions include the use of a 20 x 10 cm twin trough chamber, saturated for 20 minutes with filter paper in the rear trough and solvent level of 5 mm in each trough. The standard developing distance is 70 mm from the lower edge of the plate.

b) HPTLC of Fixed Oils

- 1st mobile phase – *Ethyl ether*,
- 2nd mobile phase: *Methylene chloride*, *glacial acetic acid* and *acetone* 20:40:50,
- Stationary phase: 20 x 10 cm, Silica gel C-18 precoated plates, 0.2 mm layer, average 4-8 um particle size,
- Band application volume: 1 µl.

Development

In the first mobile phase, develop plate upto 13 mm i.e., 5 mm above the point of application of 8 mm. Cool, air dry for 2 minutes with hair dryer. Repeat the procedure a second time.

In the second mobile phase, develop the plate upto 80 mm and dry the plate for 5 minutes in cool air with hair drier. Repeat the procedure a second time. Heat the plate on a pre heated plate heater at 120 degrees centigrade for 1 minute and examine in daylight.

Derivatization

Spray reagent: 100 mg per ml of *phosphomolybdic acid* in 96 per cent *ethanol*. Spray the plate.

Acceptance criteria: The R_f values of the principal spots of reference and sample and identical in R_f and intensity.

Drying

Drying the developed plate is best achieved while it is in the vertical position by exposure to a flow of cold air. Elevated temperature during drying could cause volatile sample components to diffuse or evaporate from the plate. During the evaporation of the mobile phase, sample components move from deeper areas of the layer to the surface. Uneven drying across the plate may result in different intensities for zones with equal amounts of substances.

Effects of Humidity

Silica gel has an extremely high affinity for water and is used as an effective drying agent. An HPTLC plate is always at equilibrium with the water vapor in the laboratory atmosphere (relative humidity, RH). Chromatographic results are affected by variation in the amount of water adsorbed onto the silica gel surface. The higher the RH, the more water is adsorbed. This reduces the "activity" of the stationary phase and causes higher R_f values. Selectivity of the chromatographic system changes as the RH changes. It is nearly impossible to predict what effect a change in RH may have for a given separation. Some separations are more affected than others, and sometimes only parts of the chromatogram will change. In addition, there is no ideal activity of the plate that can solve all separation problems, and it is difficult to use defined activity changes of the stationary phase to influence separation in a desired way. The best option is to keep the RH constant at a moderate level (e.g., 33 per cent) and then, try to adjust the selectivity of the mobile phase. One practical exemption is effective "drying off" of the plate using a molecular sieve. Activating a plate by heat (120° for 20 minutes) is possible, because under these conditions water adsorbed onto the silica gel is effectively removed, but when the plate is cooled down and stored, or during sample application it re-equilibrates to ambient relative humidity. To achieve reproducible results independent of seasonal or regional changes in the RH in various laboratories, it is important to expose the plate to a defined RH just before development and with the samples already applied. This will keep the activity and the selectivity of the stationary phase constant. Defined RH can be created in closed containers such as a desiccator or specialized equipment and over saturated salt solutions. Between 20° and 25°, saturated solutions of *magnesium chloride* establish 33 per cent RH; of *potassium thiocyanate*, 47 per cent RH; and of *sodium chloride*, 75 per cent RH.

Derivatization

Derivatization can be defined as a technique that primarily modifies analyte functionality in order to enable easy detection. Derivatization can be performed either by immersing the plates or by spraying the plates with suitable reagents carefully. For reproducible results, the amount of reagent of a

given concentration applied needs to be defined either by volume (spraying) or by volume, and dwell time (immersion). Most derivatization reactions require a heating step performed either on a plate heater or in a drying oven. The plate should be dried in air before heating to avoid any disruption of the layer resulting from evaporation of residual solvent. Before the plate is heated, the heating device should reach the required temperature. For reproducible results, it is important that the entire derivatization process is strictly timed. This includes any waiting time (e.g., for cooling) before the detection step. Detection is then performed under white light or under UV at 366 nm.

NOTE—Precaution is exercised not to char the plate while kept in oven after derivatization.

Detection by imaging of chromatogram

Generally, visualization of plates is either performed under white light, typically by using a light source above the plate (reflectance) in combination with light from below the plate (transmission), or under UV at 254 nm and 366 nm. Under UV 254 nm, the F254 indicator included in the stationary phase emits a green light (blue for F254s). Any substance that absorbs UV 254 nm will be visible as a dark "quenching" zone where the degree of quenching corresponds to the amount of that substance (and its extinction coefficient). Some substances can be excited to fluorescence by long wave UV light (UV 366 nm), particularly after chemical derivatization. Such substances are seen as zones of specific colors on the dark background. Automated computer assisted HPTLC visualizers are available for photo documentation, reproducible visualization, and evaluation of HPTLC chromatograms. In addition to visualisation under white and UV light, derivatization by specific chemical reagents, if required, is specified in the individual monographs. Selection of such derivatization reagents depends upon the nature of compounds present in the sample.

HPTLC records electronic images of chromatograms that allow convenient visual comparison of results obtained for multiple samples against images of chromatograms generated with reference materials. The HPTLC fingerprint from the same chromatogram can be evaluated in multiple detection modes, e.g., white, UV 254 nm and UV 366 nm light without derivatization, and white and UV 366 nm light after derivatization, thus enables the production of significant amount of information about the sample without the need to repeat the chromatography. Digital images can be taken of the plate in different detection/illumination modes. Parameters to acquire such images should always be the same for a given analysis regardless of the operator and location. This ensures reproducibility and elimination of variations due to human factors. The use of image editing software to change certain aspects is not permissible.

Evaluation of fingerprint image chromatograms

In the evaluation step, the analytical data obtained from samples are compared against specifications in the monograph. HPTLC for identification of herbs and herbal products typically produces fingerprints, i.e., sequences of zones that have specific positions, colors, and intensity. These fingerprints are compared against fingerprints obtained for the authenticated Indian Pharmacopoeia Botanical Reference Substance (IPBRS) and Indian Pharmacopoeia Reference Substance (IPRS). Ideally, comparison is based on images rather than descriptions. Reference and sample images are compared on one computer display. Because of the natural variability of herbal materials, there will always be a range associated with the acceptance criteria for a fingerprint to pass the identity test. Fingerprints of related herbal species or adulterants should have distinguishing features not present in samples that pass the evaluation.

Quantification

The quantitative evaluation is performed by measuring the predefined bands obtained in the sample under examination and Indian Pharmacopoeia Reference Substance (IPRS) using a scanner. External standard methods are mostly used but internal standard methods can be used. Limit tests require a single concentration standard level. The scanner needs to be set to the desired wavelength(s), either in absorption mode or in fluorescence mode (with a suitable cut-off filter in place to remove light required for excitation). Scanning at desired wavelength(s) reflects the bands in the form of peaks on area/height or on both (area and height) basis and is representative of the concentration of substances in the bands and their extinction coefficients. The software stores this data and draws the calibration curve. The beam for scanning samples is 65 to 80 per cent of the band length.

System Suitability Test

A system suitability test is required to qualify each developed plate before data obtained is considered reliable. On each plate, the analyst applies a track with 2 - 4 reference substances in defined concentrations covering the R_f range of 0.2 - 0.8. Two of these have similar but just separable R_f values under the method's chromatographic conditions. The substances are designated to check the system suitability for position, resolution, and colors of the bands after the plate is developed. Only when the system suitability requirements have been satisfied can the results obtained with the samples on the same plate be evaluated further to determine compliance. The system suitability test is of great importance especially for the identification of herbs, which have an intrinsic natural variability in chemical composition.

Documentation

Documentation of HPTLC is to be done with the help of a suitable automated system coupled to appropriate software. While doing so, samples and reference materials need to be suitably identified (numbered) and a suitable labelling system needs to be developed and adopted for proper storage/ archiving of the documents/images/profiles/densitometric data for easy retrieval in a controlled quality assurance system and for auditing.

Apparatus

The equipment used for HPTLC technique consists of the following:

- Plates. Unless otherwise specified in the individual monograph, use plates coated with a uniform 200-µm layer of porous (60 Å pore size) silica gel having irregular particles of 2–10 µm and an average particle size of 5 µm, a polymeric binder, and a fluorescence indicator (F254) of 20 × 10 cm. [*NOTE—Chromatographic methods using high-performance thin-layer chromatographic glass plates are preferred over aluminum-backed sheets because of greater mechanical stability*].
- A device suitable for the application of specified volumes of samples as bands with specified length at the specified positions.
- A suitable chromatographic chamber (for example, a twin trough chamber) allowing for control of saturation and developing distance.
- A device suitable for controlling the activity of the stationary phase via relative humidity.
- A device suitable for reproducible drying of the developed plate.
- A device suitable for treatment of the plate with derivatization reagent, if required.
- A device suitable for heating as part of the derivatization procedure, if required.
- A system suitable for documentation of chromatograms under UV 254 nm, UV 366 nm, and white light.
- A suitable HPTLC software to integrate the instruments into one HPTLC system, store and process the analytical information.
- A scanner suitable for scanning plates for quantitative analysis.

Each of these devices as well as the system as a whole should pass installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) in order to assure that the instruments are working according to their specifications.

HPTLC Standard Conditions (applicable unless specified otherwise in the individual monograph)

Plate. 20 × 10 cm or 10 × 10 cm glass or aluminum foil plate with HPTLC Silica gel 60 F 254 and polymeric binder.

Plate layout. Bands of 6-8 mm length, 8 mm from lower edge of plate, minimum 2 mm apart, center of first band about 20 mm from the left edge of plate and similarly on right edge.

Plate conditioning. A 33 per cent RH immediately before development.

Chamber. 20 × 10 cm twin trough chamber, solvent level of 5 mm in both troughs, 20 minutes saturation with filter paper in the rear trough, developing distance 70 mm from lower edge of plate.

Drying. 5 minutes in a stream of cold air, immediately after removal from chamber. Plate held vertically.

Image Documentation. Under UV 254 nm; UV 366 nm, and white light prior to derivatization, under UV 366 and white light after derivatization.

Densitometric scanning for quantification: A scanner for plates, with UV and Visible range, suitable for fluorescence measurements, monochromator, variable scanning slit sizes, different calibration modes for quantitative analysis.

2.4.18. Jelly Strength

The Jelly strength of Gelatin is the weight, in g, necessary to give a 4-mm depression in a jelly containing 6.67 per cent w/w, matured at 10°, using a plunger 12.7 mm in diameter.

Apparatus

A gelometer consisting of a cylindrical piston 12.6 to 12.8 mm in diameter with a plane pressure surface with a rounded edge 0.5 mm in radius attached to a device whereby the load exerted by the piston can be increased at a constant rate of 40 g per second and the vertical movement of the piston can be stopped within 0.025 seconds when it has descended 3.9 to 4.1 mm.

Method

Place 7.5 g of the substance under examination in a bottle, 58 to 60 mm in internal diameter and 85 mm high, add 105 ml of *water*, cover the bottle with a watch glass and allow to stand for 3 hours. Heat in a water-bath at 65° for 15 minutes, stirring gently with a glass rod ensuring that the solution is uniform and that any condensed water on the inner walls of the bottle is incorporated. Allow to cool at room temperature for 15 minutes, transfer to a water-bath maintained at 9.9° to 10.1° and ensure that the base of the bottle is horizontal. Close the bottle with a rubber stopper and allow to stand for 16 to 18 hours. Immediately transfer the bottle to the gelometer and

adjust the height of the bottle so that the piston just comes into contact with the surface of the gel without exerting any pressure. Increase the load on the piston at a rate of 40 g per second until it has descended 3.9 to 4.1 mm. The load, measured within a precision of ± 0.5 g, exerted by the piston at that moment represents the jelly strength. Carry out five determinations and use the mean value.

2.4.19. Loss on Drying

Loss on drying is the loss of weight expressed as percentage w/w resulting from *water* and volatile matter of any kind that can be driven off under specified conditions. The test is carried out on a well-mixed sample of the substance. If the substance is in the form of large crystals, reduce the size by rapid crushing to a powder.

Where the drying temperature is indicated by a single value other than a range, drying is carried out at the prescribed temperature ± 2°.

Unless otherwise specified in the individual monograph, use Method A.

Method A

Weigh a glass-stoppered, shallow weighing bottle that has been dried under the same conditions to be employed in the determination. Transfer to the bottle the quantity of the sample specified in the individual monograph, cover it and accurately weigh the bottle and the contents. Distribute the sample as evenly as practicable by gentle sidewise shaking to a depth not exceeding 10 mm.

Dry the substance by placing the loaded bottle in the drying chamber as directed in the monograph, remove the stopper and leave it also in the chamber. Dry the sample to constant weight or for the specified time and at the temperature indicated in the monograph. Dry by one of the following procedures. After drying is completed, open the drying chamber, close the bottle promptly and allow it to cool to room temperature (where applicable) in a desiccator before weighing. Weigh the bottle and the contents.

a) "in a desiccator": dry over *phosphorus pentoxide* at atmospheric pressure and at room temperature;

NOTE — Care must be taken to keep the desiccant fully effective by frequent replacement.

b) "in vacuo": dry over *phosphorus pentoxide*, at a pressure of 1.5 kPa to 2.5 kPa at room temperature;

c) "in vacuo within a specified temperature range": dry over *phosphorus pentoxide*, at a pressure of 1.5 kPa to 2.5 kPa within the temperature range given in the monograph;

d) "in an oven within a specified temperature range": dry in an oven within the range given in the monograph;

NOTE — Where the drying temperature is indicated by a single value, dry at the prescribed temperature ± 2°.

e) "under high vacuum": dry over *phosphorus pentoxide*, at a pressure not exceeding 0.1kPa, at the temperature given in the monograph.

Method B

Thermogravimetry. Thermogravimetry is a technique in which the weight of a sample is recorded as a function of temperature according to a controlled temperature programme.

Apparatus

A thermobalance consisting of a device for heating or cooling the substance being examined according to a given temperature programme, a sample holder in a controlled atmosphere, an electrobalance and a recorder. The instrument may be coupled to a device permitting the analysis of volatile products.

Temperature verification. Check the temperature scale using nickel or other suitable material according to the manufacturer's instruction.

Calibration of the electrobalance

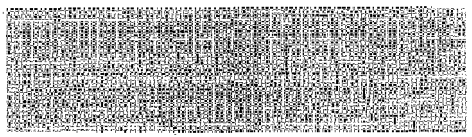
Place a suitable quantity of *calcium oxalate monohydrate RS* in the sample holder and record the weight. Set the heating rate according to the manufacturer's instructions and start the temperature programme. Record the thermogravimetric curve as a graph with temperature on the abscissa, increasing from left to right, and weight on the ordinate, increasing upwards. Stop the rise in temperature at 230°. Measure the distance on the graph between the initial and final weight-temperature plateaux that corresponds to the loss of weight. The declared loss of weight for *calcium oxalate monohydrate RS* is stated on the label.

NOTE — If the apparatus is in frequent use, carry out temperature verification and calibration regularly. Otherwise, carry out such checks before each measurement.

Procedure

Apply the same procedure to the substance under examination, using the conditions prescribed in the monograph. Calculate the loss of weight of the substance under examination from the distance measured on the graph obtained and express as a percentage w/w of the substance taken.

The actual procedure and the calculations to be employed are dependent on the particular instrument used. Consult the manufacture's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism and decomposition during the analysis.



2.4.20. Loss on Ignition

Loss on ignition is the loss in weight in per cent w/w resulting from a part of any test material, that is volatilised and driven off under specified conditions. The test is performed on finely powdered material; lumps, if any should be broken up with the aid of a mortar and pestle.

Method

Weigh a silica or platinum crucible, cover with the lid, previously ignited for 1 hour at the temperature specified for the test and cooled in a desiccator. Transfer to the crucible the quantity of the substance specified in the individual monograph, without any treatment, unless a preliminary drying at a lower temperature, or other special treatment is specified. Weigh accurately the crucible, lid and the contents. Place the loaded uncovered crucible and cover in a suitable muffle furnace or oven that is capable of maintaining a temperature within 25° of that required for the test. Ignite the crucible for the period of time and at the temperature stated in the monograph. Ignite for successive 1-hour periods where ignition to constant weight is indicated. Upon the completion of each ignition, cover the crucible and allow it to cool in a desiccator to room temperature before weighing.

2.4.21. Melting Range or Temperature

In this Pharmacopoeia, melting range or temperature of a substance is defined as those points of temperature within which, or the point at which, the substance begins to coalesce and is completely melted except as defined otherwise for certain substances. The following procedures are suitable for the various substances described in the Pharmacopoeia. Any other apparatus or method capable of the same accuracy may also be used. The accuracy should be checked frequently by using certified reference substances of declared melting point, such as those of the World Health Organization or other suitable substances, the reference substance selected being one that melts nearest to the melting range of the substance to be examined.

Unless otherwise specified in the individual monograph, use Method I.

Method I

Apparatus

(a) A glass heating vessel of suitable construction and capacity containing one of the following or any other suitable bath liquid, to a height of not less than 14 cm.
   (i) Water for temperatures upto 60°.
   (ii) Glycerin for temperatures upto 150°.
   (iii) Liquid paraffin of sufficiently high boiling range for temperatures upto 250°.
   (iv) Sesame oil or a suitable grade of silicone oil for temperatures upto 300°.

(b) A suitable stirring device capable of rapidly mixing the liquids.

(c) An accurately standardised thermometer suitable for the substance under examination (see Appendix 2.1.4). The thermometer must be positioned in the bath liquid to its specified immersion depth and yet leave the bulb about 2 cm above the bottom of the bath.

(d) Thin-walled capillary glass tubes of hard glass, closed at one end, about 12 cm long, with a thickness of 0.2 to 0.3 mm and an internal diameter of 0.8 to 1.1 mm. The tubes should preferably be kept sealed at both ends and cut as required. A suitable magnifying glass may be used for observation of melting in the capillary tube.

(e) A source of heat (open flame or electric heater).

Procedure

Reduce the substance to a very fine powder and, unless otherwise directed, dry it at a temperature considerably below its melting temperature or at a pressure of 1.5 to 2.5 kPa over *self-indicating silica gel* for 24 hours. Introduce into a capillary glass tube, a sufficient quantity of the dry powder to form a compact column 4 to 6 mm high. Heat the bath until the temperature is about 10° below the expected melting temperature. Remove the thermometer and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise and adjust its height so that the closed end of the capillary is near the middle of the thermometer bulb. Replace the thermometer and continue the heating, with constant stirring, sufficiently to cause the temperature to rise at a rate of about 10° per minute. Continue the heating and note the temperature at which the column of the sample collapses definitely against the side of the tube at any point, when melting may be considered to have begun and note also the temperature at which the sample becomes liquid throughout as seen by the formation of a definite meniscus. The two temperatures fall within the limits of the melting range.

Method II

Apparatus

Use the apparatus described under Method I except that the glass capillary tube is open at both ends and has an internal diameter of 1.1 to 1.3 mm, an external diameter of 1.4 to 1.7 mm and length of 50 to 60 mm.

Procedure

Rapidly melt the substance under examination, at a temperature not more than 10° above the point of complete fusion. Draw it

into a capillary tube to a depth of about 10 mm. Cool the charged tube at 10° or lower for 24 hours, or keep in contact with ice for at least 2 hours. Attach the tube to the thermometer and adjust it so that the column of the substance under examination is in level with the thermometer bulb. Suspend the thermometer in the heating vessel containing water at 15° so that the lower end of the column of the substance is 30 mm below the surface of the water and heat the water with constant stirring so that the temperature rises at the rate of 1° per minute. The temperature at which the partly melted substance is observed to rise in the capillary tube is the melting temperature.

Method III

Apparatus

(a) A glass boiling-tube of overall length 110 mm and internal diameter 25 mm.

(b) A cork about 25 mm long to fit into the boiling-tube, bored with a central hole to fit a standard thermometer and with a groove cut in the side.

(c) A glass beaker of such a size that when the apparatus is assembled the boiling-tube can be immersed vertically to two-third of its length in the water present in the beaker with its lower end about 2.5 cm above the bottom of the beaker.

(d) A stirrer or any other device which will ensure uniformity of the temperature throughout the water in the beaker.

(e) An accurately standardised thermometer suitable for the substance under examination (see Appendix 2.1.4).

(f) Suitable means for heating the water in the beaker.

Procedure

Melt a quantity of the substance under examination slowly, while stirring, until it reaches a temperature of about 90°. Cool and allow the temperature of molten substance to drop to a temperature 8° to 10° above the expected melting temperature. Chill the bulb of the thermometer to 5°, wipe it dry and while it is still cold dip it in the molten substance so that the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 minutes into a water bath at a temperature not higher than 15°.

Fit the thermometer through the bored cork into the boiling-tube so that the lower part is 15 mm above the bottom of the tube. Suspend the tube in the beaker filled with water adjusted to about 15° and raise the temperature of the bath to 30° at the rate of 2° per minute, adjust the rate to 1° per minute and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the substance. If the three readings differ by less than 1°, take the average of the three as the melting temperature. If they differ by more than 1°, make two additional determinations and take the average of the five readings.

Method IV

Apparatus

The apparatus (see Fig. 2.4.21-1) consists of the following components:

(a) An accurately standardised thermometer calibrated for 100 mm immersion, covering the range -5° to +105°, and conforming to Indian Standard 4825:1968 but with the following modifications:

Bulb diameter	3.35 to 3.65 mm
Bulb length	not greater than 5 mm

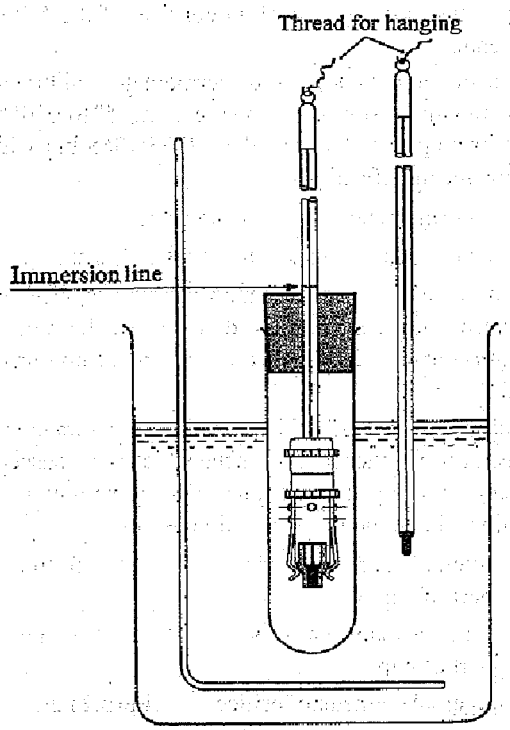
Stem diameter, immediately above the bulb, approximately equal but not less than the diameter of the bulb for a length of at least 26 mm measured from the bottom of the bulb.

b) A metal cup (see Fig 2.4.21-1a) made from chromium-plated brass or from other suitable metal which is not susceptible to corrosion by the substance under examination and conforming to the following dimensions:

Internal diameter of wide part of cup	7.35 to 7.65 mm
External diameter of wide part of cup	9.95 to 9.99 mm
Internal diameter of orifice	3.1 to 3.2 mm
External diameter of orifice	5.5 to 5.6 mm
Overall length of cup	15.0 to 15.4 mm

Internal depth of wide part of the cup: The lower part of the wide portion of the cup is approximately part of a hemisphere. When a steel ball, 7.00 mm in diameter is placed in the cup, the top of the ball is 12.05 to 12.35 mm above the bottom of the tube forming the orifice. The top of the cup and the bottom of the tube forming the orifice are smooth, parallel to each other and at right angles to the axis of the cup. The bottom edge of the orifice is not chamfered or radiused.

c) To the lower end of the thermometer is securely and coaxially cemented with a cylindrical metal sleeve on to which a metal case is screwed (see Figs 2.4.21-1b and 2.4.21c). This metal case has two small apertures, one in front and one behind, to act as air vents. It also has a rim stop, guide lugs to aid the coaxial insertion of the cup into the case and spring clips to enable the cup to be retained firmly with its top edge against the rim stop. The metal sleeve is fixed to the thermometer in such a position that, when the metal case is screwed on to the sleeve, the bottom of the thermometer bulb is 7.9 to 8.2 mm below the rim stop.



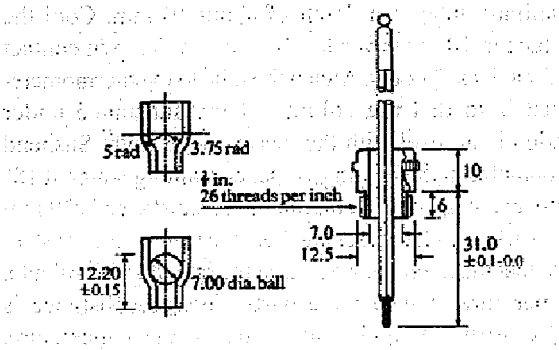
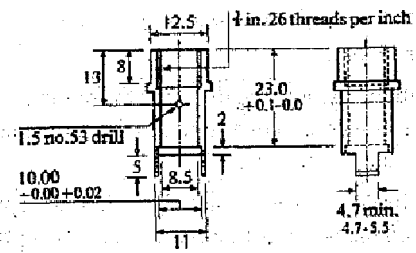


Fig. 2.4.21-1b
Relative position of
Thermometer and sleeve



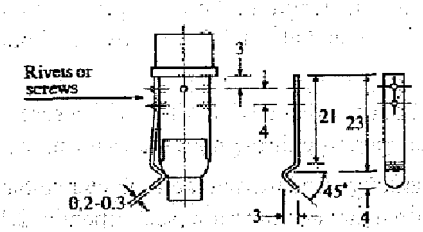


Fig. 2.4.21-1c
Cup

General assembly *(Dimensions in mm)*

Fig. 2.4.21-1: Apparatus for Determination of Melting Range or Temperature

d) Auxiliary apparatus assembled as shown in Fig 2.4.21-1 and consisting of the following parts.

(i) A glass tube, overall length 110 mm, internal diameter 25 mm.

(ii) A cork about 25 mm long, bored with a central hole to fit the standard thermometer and with a groove cut in the side.

(iii) A glass beaker, of such size that when the apparatus is assembled the boiling tube can be immersed vertically to two third of its length in the liquid in the beaker with its lower end about 25 mm above the bottom of the beaker. The liquid used in the beaker should be water for melting points (flow and drop points) below 80° and liquid paraffin or glycerin for melting points above 80°.

(iv) A stirrer or any other convenient device that will ensure uniformity of temperature throughout the liquid in the beaker.

(v) An auxiliary thermometer.

(vi) A stand with claimps for holding the boiling tube and auxiliary thermometer in position in the beaker and for holding the beaker above the source of heat.

(vii) A suitable means of heating the water in the beaker.

Procedure

Heat the sample, with stirring, to 118° to 122°, to ensure uniformity, and then cool to 103° to 107°. Warm the metal cup to 103° to 107° in an oven, remove it from the oven, place on a clean plate or ceramic tile and pour sufficient of the melted sample into the cup to fill it completely. Allow the filled cup to cool for 30 minutes on the tile or plate and then place it in a

water-bath at 24° to 26° for a further 30 to 40 minutes. Level the surface of the sample with a single stroke of a knife or razor blade, avoiding any stirring of the sample. Push the cup, without lateral movement, into the metal case as far as the rim stop and wipe away the excess of the substance that is squeezed out of the bottom of the tube, ensuring that the air vents are not blocked. Fit the thermometer, with the cup attached, through the bored cork to the boiling tube such that the bottom of the cup is 24 to 26 mm above the bottom of the boiling tube. Fix the boiling tube vertically within the beaker so that at least two thirds of its length is immersed in the liquid contained in the beaker. Adjust the temperature of the outer bath so that the temperature of the substance rises at the rate of 1° per minute. The temperature at which the first drop of melted liquid falls from the metal cup is regarded as the melting point (drop point) of the substance. Note the temperature at the fall of the first drop. Carry out at least three determinations, each time with a fresh sample of the substance under examination. The difference between the readings must not exceed 3°. The mean of three readings is taken as the melting point of the substance.

2.4.22. Optical Rotation and Specific Optical Rotation

Optical rotation, 'α' is the property shown by chiral substances of rotating the plane of polarisation of linearly polarized light. Such substances are said to be optically active in the sense that they cause incident polarised light to emerge in a plane forming a measurable angle with a plane of the incident light. Where this effect is large enough for measurement, it may serve as the basis for identifying or assaying a substance.

The optical rotation of a substance is the angle through which the plane of polarisation is rotated when polarised light passes through the substance, if liquid, or a solution of the substance. Substances are described as *dextro*-rotatory or *levo*-rotatory according to whether the plane of polarisation is rotated clockwise or anticlockwise, respectively as determined by viewing towards the light source. *Dextro*-rotation is designated (+) and *levo*-rotation is designated (-).

The optical rotation, unless otherwise specified, is measured at the wavelength of the D line of sodium (λ = 589.3 nm) at a temperature 25°, on a layer 1 dm length. It is expressed in degrees.

The specific optical rotation, $[\alpha]_D^{25}$, of a liquid substance is the angle of rotation, 'α', of the plane of polarisation at the wavelength of the D line of sodium (λ = 589.3 nm) measured at a temperature 25°, unless otherwise specified, calculated with reference to a 1-dm thick layer of the liquid, and divided by the specific gravity at a temperature 25°.

The specific optical rotation, $[\alpha]_D^{25}$, of a solid substance is the angle of rotation, 'α', of the plane of polarisation at the wavelength of the D line of sodium (λ = 589.3 nm) measured at a temperature 25°, unless otherwise specified, calculated with reference to a 1-dm thick layer of a solution containing 1 g of the substance per 100 ml. The specific optical rotation of a solid is always expressed with reference to a given solvent and concentration.

NOTE — Some equipment may not use sodium lamps, the wavelength of measurement is given as 589 nm instead of 589.3 nm.

Apparatus

A commercial instrument constructed for use with a sodium lamp and capable of giving readings to the nearest 0.01° is suitable for most purposes. For certain applications, the use of a photoelectric polarimeter capable of taking measurements at the specific wavelengths may be necessary.

The accuracy and precision of optical rotation measurements can be increased if the following precautions are taken.

(a) The instrument must be in a good condition. The optical elements must be very clean and in exact alignment. The match point should be close to the normal zero mark.

(b) The light source should be properly aligned with respect to the optical bench. It should be supplemented by a filtering system capable of isolating the D line from sodium light.

(c) Specific attention should be paid to temperature control of the solution and of the polarimeter. The polarimeter may consists of a temperature control system that indicates the temperature with a readability of 0.1°; it may be embedded in the polarimeter (e.g. a Peltier system) or be an external unit (e.g. a cycle-cryostat), and must be able to maintain the temperature of the liquid to within ± 0.5° of that prescribed.

(d) Differences between the initial readings or between observed and corrected optical rotation, calculated as either specific optical rotation or optical rotation, should not be more than one-fourth of the range specified in the monograph for the substance.

(e) Polarimeter tubes should be filled in such a way as to avoid air bubbles. Particular care is necessary for semi-micro or micro tubes.

(f) For tubes with removable end-plates fitted with gaskets and caps, tighten the end-plates only enough to ensure a leak-proof seal between the end-plate and the body of the tube.

(g) For substances with low rotatory power, the end-plates should be loosened and tightened again after each reading, in the measurement of both the rotation and the zero point.

(h) Liquids and solutions of solids must be clear.

Calibration

The accuracy of the scale may be checked near the value to be measured or over an appropriate range, usually by means of certified quartz plates or certified sucrose for calibration.

Optical rotation measurements may be used to quantify the amount of an enantiomer or the ratio of enantiomers present in a sample and for that purpose, the linearity must be checked.

Sucrose solution

The apparatus may be checked by using sucrose solution of various concentrations as indicated in Table- 1. Use sucrose reference material, previously dried at 105° for 3 hours. Measure the optical rotation of the solutions in 1-dm tube at 25°. Note the angle of rotation for solutions of all concentrations and perform the regression analysis to calculate the Correlation Coefficient (r). Calculate the specific optical rotation (SOR) as per acceptance criteria.

Table

Concentration (g per100 ml)	Angle of Rotation (+) at 25°
10.0	6.67
20.0	13.31
30.0	19.93
40.0	26.53
50.0	33.12

Acceptance criteria

— The correlation coefficient (r) should not be less than 0.999.

— Specific optical rotation of 10 per cent w/v solution should be between + 66.0° to + 67.4°.

Method

For solids — Weigh accurately a suitable quantity of the substance under examination to obtain the solution of the strength specified in the individual monograph and transfer to a volumetric flask by means of water or other solvent, if specified. If a solvent is used, reserve a portion of it for the blank determination. Unless otherwise specified, adjust the contents of the flask to 25° ± 0.5° by suspending the flask in a constant-temperature bath. Make up the volume with the solvent at a temperature 25° and mix well. Transfer the solution to the polarimeter tube within 30 minutes from the time the substance was dissolved and during this time interval maintain the solution at a temperature 25° ± 0.5°.

Determine the zero point of the polarimeter and then make five readings of the observed rotation of the test solution at a temperature of 25°. Take an equal number of readings in the same tube with the solvent in place of the test solution. The zero correction is the average of the blank readings, and is subtracted from the average observed rotation if the two figures are of the same sign or added if they are opposite in sign to obtain the corrected observed rotation.

For liquids — Unless otherwise specified, adjust the temperature of the substance under examination to 25°, transfer to a polarimeter tube and proceed as described For solids, beginning at the words "Determine the zero point...".

Calculations

Calculate the specific optical rotation using the following formulae:

For liquids $[\alpha]_D^{25} = \alpha / ld^{25}$

For solids $[\alpha]_D^{25} = 100\alpha / lc$

where α = corrected observed rotation, in degrees, at a temperature of 25°,

D = D line of sodium light (l = 589.3 nm),

l = length of the polarimeter tube in dm,

d_{25}^{25} = specific gravity of the liquid or solution at 25°,

c = concentration of the substance in per cent w/v.

NOTE — The requirement for specific optical rotation in the Pharmacopoeia apply to a dried, anhydrous or solvent-free material in all those monographs in which standards for loss on drying, water or solvent content respectively are given. In calculating the result, the loss on drying, water or solvent content determined by the method specified in the monograph is taken into account.

2.4.23. Osmolality

Osmolality is a practical means of giving an overall measure of the contribution of the various solutes present in a solution to the osmotic pressure of the solution.

The expression of concentration of electrolytes in terms of mass concentrations such as mg per litre is of little clinical value. The earlier practice of expressing in terms of milliequivalents (mEq) per litre has now been replaced by molar or more correctly, the osmolar concentrations of electrolyte solutions and parenteral fluids. Knowledge of the osmolar concentration indicates whether a solution is hypo-osmotic, iso-osmotic or hyper-osmotic.

A quantitative statement of osmolar concentration facilitates calculation of the dilution required to render a hyper-osmotic solution iso-osmotic. It also simplifies many calculations involved in peritoneal dialysis and haemodialysis procedure.

Table

Wt. in g of sodium chloride per kg of water	Real osmolality (mOsmol/kg)	Ideal osmolality (mOsmol/kg)	Molal osmolality Coefficient	Cryoscopic depression (°C)
3.087	100	105.67	0.9463	0.186
6.260	200	214.20	0.9337	0.372
9.463	300	323.83	0.9264	0.558
12.684	400	434.07	0.9215	0.744
15.916	500	544.66	0.9180	0.930
19.147	600	655.24	0.9157	1.116
22.380	700	765.86	0.9140	1.302

Osmolality is a means of measuring the contribution of the various solutes present in a solution to the osmotic pressure of the solution.

The unit of osmolality is osmole per kilogram (osmol/kg). An osmol is defined as the weight in grams of a solute, existing in solution as molecules (and/or ions, macro-molecules, aggregates etc.) that is osmotically equivalent to the gram-molecular-weight (mole) of an ideally behaving nonelecrolyte. Thus the osmol-weight of a nonelectrolyte, in a dilute solution, is generally equal to its gram-molecular weight. A milliosmol, abbreviated as mOsmol, is the weight stated in milligrams and is the unit usually used.

The units of osmolar concentration are usually expressed as milliosmols (mOsmols) of solute per litre of solution. The osmolar concentration may be calculated from one of the following equations

For a nonelectrolyte:

$$\frac{\text{g/litre}}{\text{mol.wt.}} \times 1000 = \text{mOsmol/litre}$$

For strong electrolyte:

$$\frac{\text{g/litre}}{\text{mol.wt.}} \times \text{number of ions formed} \times 1000 = \text{mOsmol / litre}$$

For individual ions, if desired:

$$\frac{\text{g of ion/litre}}{\text{ionic wt.}} \times 1000 = \text{mOsmol (of ion)/litre}$$

These are simple calculations; however, they omit consideration of factors such as solvation and inter-ionic forces. By this method of calculation, a 0.9 per cent w/v solution of *sodium chloride* has an osmolar concentration of 308 mOsmols per litre (9/58.4 x 2 x 1000). This is the ideal osmolar concentration. In fact, the number of ions is slightly less than 2 for solutions of sodium chloride at this concentration, and the actual measured osmolarity of 0.9 per cent w/v Sodium Chloride Injection is about 286 mOsmols per litre. In general, as the concentration of the solute increases, interaction among solute particles increases and the actual osmolar values decrease when compared to the ideal values. Deviation from ideal conditions is usually slight in solutions within the physiologic range and for more dilute solutions, but for highly concentrated solutions the actual osmolarities may be appreciably lower than ideal values.

The derivation of the osmolar concentrations from the stated composition of the solution may be verified by calculations using the equations given above. The osmolarity of a mixture of complex composition such as Protein Hydrolysate Injection cannot be readily and confidently calculated. In such instance, actual values of osmolar concentration are determined by calculating the osmolarity from measured values of osmolal concentration and water content. Each osmol of solute added to 1 kg of water lowers the freezing point approximately 1.86° and lowers the vapour pressure approximately 0.3 mm of mercury at 25°. These physical changes are measurable and they permit accurate measurements of osmolal concentrations.

Unless otherwise directed, osmolality is determined by measurement of the depression of freezing point ΔT. The osmolality (ξ_m) in mosmol per kg is calculated from the expression

$$\xi_m = \frac{\Delta T}{1.86} \times 1000 \text{ mOsmol/kg}$$

Apparatus. It consists of a system of cooling the container used for the measurement, an arrangement for mixing the sample, and a system for measuring the temperature by means of a device for determining the current or potential-difference, graduated in temperature depression or directly in osmolality.

Method. Prepare the required reference solutions given in the table. Determine the zero of the apparatus using water. Calibrate the apparatus using the reference solutions in the following

manner. Introduce 50 µl to 250 µl of the sample into the measurement cell and start the cooling. Operate the cooling device at a temperature below that expected through cryoscopic depression to prevent supercooling. When equilibrium is attained record the freezing point. Before each measurement, rinse the measurement cell with the solution to be examined. The instrument is calibrated by using two standard solutions of sodium chloride that span the expected range of osmolarities.

Carry out the same operations with the test sample. Read directly the osmolality or calculate it from the measured depression of freezing point. The test is not valid unless the value found is within two values of the calibration scale.

2.4.24. pH Values

The pH value conventionally represents the acidity or alkalinity of an aqueous solution. In the Pharmacopoeia, standards and limits of pH have been provided for those pharmacopoeial substances in which pH as a measure of the hydrogen-ion activity is important from the standpoint of stability or physiological suitability. The determination is carried out at a temperature of 25° ± 2°, unless otherwise specified in the individual monograph.

Apparatus

The pH value of a solution is determined potentiometrically by means of a glass electrode, a reference electrode and a pH meter either of the digital or analogue type.

Operate the pH meter according to the manufacturer's instructions. Calibrate the apparatus using *buffer solution D* as the primary standard, adjusting the meter to read the appropriate pH value given in the table, corresponding to the temperature of the solution. To set the scale, use a second reference buffer solution, either *buffer solution A*, *buffer solution E* or *buffer solution G* and carry out a check with a third buffer solution of intermediate pH. The pH reading of the intermediate solution must not differ by more than 0.05 from the corresponding value indicated in the table.

Reference Buffer Solutions

NOTE — Prepare the following buffer solutions using carbon dioxide-free water. Buffer solutions should be stored in bottles made of alkali-free glass, and must not be used later than 3 months after preparation.

A. A 1.271 per cent w/v solution of *potassium tetraoxalate*.

B. A freshly prepared solution, saturated at 25°, of *potassium dihydrogen tartrate*.

C. A freshly prepared 1.151 per cent w/v solution of *potassium dihydrogen citrate*.

D. A 1.021 per cent w/v solution of *potassium hydrogen phthalate*, previously dried at 110° to 135° for 2 hours.

E. A mixture containing 0.348 per cent w/v of *potassium dihydrogen phosphate* and 0.355 per cent w/v of *anhydrous disodium hydrogen phosphate*, both previously dried at 110° to 130° for 2 hours.

F. A mixture containing 0.1184 per cent w/v of *potassium dihydrogen phosphate* and 0.4303 per cent w/v of *anhydrous disodium hydrogen phosphate*, both previously dried at 110° to 130° for 2 hours.

G. A 0.3814 per cent w/v solution of *sodium tetraborate* stored protected from carbon dioxide.

H. A mixture containing 0.2649 per cent w/v of *sodium carbonate* and 0.210 per cent w/v of *sodium bicarbonate*.

I. Shake an excess of *calcium hydroxide* with *carbon dioxide-free water* and decant at 25°.

Method

Immerse the electrodes in the solution under examination and measure the pH at the same temperature as for the standard solutions. At the end of a set of measurements, record the pH of the solution used to standardise the meter and the electrodes. If the difference between this reading and the

Table - pH of reference buffer solutions at various temperatures

Temperature	Reference Buffer Solution								
t°	A	B	C	D	E	F	G	H	I
15	1.67	–	3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68	–	3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
ΔpH/Δt	+0.001	−0.0014	−0.0022	+0.0012	−0.0028	−0.0028	−0.0082	−0.0096	−0.034

original value is greater than 0.05, the set of measurements must be repeated.

When measuring pH values above 10.0 ensure that the glass electrode is suitable for use under alkaline conditions and apply any correction that is necessary.

All solutions and suspensions of substances under examination must be prepared using *carbon dioxide-free water*.

2.4.25. Titrimetry

Titrimetry is a procedure of analysis where according to the kind of reaction and nature of the phenomenon occurring between the titrant and the titrate classified into Direct, Residual, Complexometric, Oxidation-Reduction (Redox) Non-aqueous titration and Potentiometrically Endpoint Detection.

Blank Corrections. The endpoint determination in a Titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the Titrant. An appropriate blank correction is employed in Titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a residual blank titration, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of Titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under Residual Titrations. Where potentiometrically endpoint detection is employed, the blank correction is usually negligible.

Direct Titrations. Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the Titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator. The Titrant is added from a suitable burette and is so chosen, with respect to its strength (normality), that the volume added is between 30 per cent and 100 per cent of the rated capacity of the burette. (Where less than 10 of Titrant is required, a suitable microburst is to be used.) The endpoint is approached directly but cautiously, and finally the Titrant is added drop wise from the burette in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the Titrant and the equivalence factor for the substance as specified in the individual monograph.

Residual Titrations. Some Pharmacopoeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the Titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

Complexometric Titrations. Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the Titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100 per cent of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful. In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or "screened" via addition of another complexing agent. (The masking technique is also applicable to redox titrations).

Oxidation-Reduction (Redox) Titrations. Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst's ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate. In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or

reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.

Titrations in Non-aqueous Solvents. Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is *perchloric, hydrobromic, sulphuric, hydrochloric,* and *nitric* (differentiating effect). *Acetic acid* reacts incompletely with *water* to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as *ethylenediamine*, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for *perchloric acid.*

This leveling effect is observed also for bases. In *sulphuric acid* almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series *sulphuric acid, acetic acid, phenol, water, pyridine,* and *butylamine,* the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are *sodium 2-aminoethoxide, potassium methoxide, sodium methoxide,* and *lithium methoxide.* Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of *mercuric acetate,* which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of *perchloric acid* in *glacial acetic acid* is preferred, although *perchloric acid* in *dioxane* is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of *methanol* and *toluene* is used frequently, although *lithium methoxide* in *methanol-benzene* solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide. The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., *tetra-n-butylammonium hydroxide* and *trimethylhexadecylammonium hydroxide* (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 of *0.1 M sodium methoxide* of solvent. The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with *0.1 M lithium perchlorate* in *glacial acetic acid* for titrations in acidic solvents or *potassium chloride* in *methanol* for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the *aqueous potassium chloride solution* and

residual potassium chloride, if any, by rinsing with *water*, then eliminating residual *water* by rinsing with the required non-aqueous solvent, and finally filling the electrode with the designated non-aqueous mixture. In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in non-aqueous solvents are listed in Table 1.

Indicator and Potentiometrically Endpoint Detection. The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus of Titrant added; for a precipitation, complexometric, or oxidation-reduction titration, mV versus of Titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the endpoint. The

Table-1. Systems for Non-aqueous Titrations

Type of	Acidic (for titration of bases and their salts)	Relatively Neutral (for differential titration of bases)	Basic (for titration of acids)	Relatively Neutral (for differential titration of acids)
Solvent[1]	Glacial Acetic Acid	Acetonitrile	Dimethylformamide	Acetone
	Acetic Anhydride	Alcohols	*n*-Butylamine	Acetonitrile
	Formic Acid	Chloroform	Pyridine	Methyl Ethyl Ketone
	Propionic Acid	Benzene	Ethylenediamine	Methyl Isobutyl Ketone
	Sulphuryl Chloride	Toluene	Morpholine	*tert*-Butyl Alcohol
		Chlorobenzene		
		Ethyl Acetate		
		Dioxane		
Indicator	Crystal Violet	Methyl Red	Thymol Blue	Azo Violet
	Quinaldine Red	Methyl Orange	Thymolphthalein	Bromothymol Blue
	p-Naphtholbenzein	*p*-Naphtholbenzein	Azo Violet	*p*-Hydroxyazobenzene
	Alphazurine 2-G		*o*-Nitroaniline	Thymol Blue
	Malachite Green		*p*-Hydroxyazobenzene	
Electrodes	Glass–calomel	Glass–calomel	Antimony–calomel	Antimony–calomel
	Glass–silver–silver chloride	Calomel–silver–silver chloride	Antimony–glass	Glass–calomel
	Mercury–mercuric acetate		Antimony–antimony[2]	Glass–platinum[2]
			Platinum–calomel	
			Glass–calomel	

[1]Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.

[2]In titrant.

Table-2. Potentiometrically Titration Electrode Systems

Titration	Indicating Electrode	Equation[1]	Reference Electrode	Applicability[2]
Acid-base	Glass	$E = k + 0.0591\ pH$	Calomel or silver-silver chloride	Titration of acids and bases
Precipitimetric (silver)	Silver	$E = E^{\circ} + 0.0591 \log [Ag^{+}]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Complexometric	Mercury–mercury(II)	$E = E^{\circ} + 0.0296$ (log k _ pM)	Calomel	Titration of various metals (M), e.g., Mg^{+2}, Ca^{+2} Al^{+3}, Bi^{+3}, with EDTA
Oxidation–reduction	Platinum	$E = E^{\circ} + (0.0591/n) \times \log [ox]/[red]$	Calomel or silver–silver chloride	Titrations with arsenite, bromine, cerate, dichromate, exacyonoferrate(III), iodate, nitrite, permanganate, thiosulfate

[1]Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation:

$ox + ne \leftrightarrow red$.

[2]Listing is representative but not exhaustive.

equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometrically endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction

$$2Ag^{+} + CrO_4^{-2}$$

and the oxidation-reduction reaction

$$5Fe^{+2} + MnO_4^{-}$$

All acid-base reactions, however, are symmetrical. Thus, potentiometrically endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out Titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, Titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the Titrant addition ceases.

Several acceptable electrode systems for potentiometrically titrations are summarized in Table 2.

2.4.26. Solubility

NOTE — A test for solubility becomes a test for purity only where a special quantitative test is given in the individual monograph and is an official requirement.

The approximate solubilities of the articles of the Pharmacopoeia are given here primarily as information; they are not meant to be applied as tests for identifying materials. However, they may indirectly help in the preliminary evaluation of the integrity of an article. They have been indicated by descriptive terms in the accompanying table and have the following significance with reference to a temperature of 15° to 30°.

Descriptive term	Parts of Solvent required for Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble, or Insoluble	10,000 or more

The term 'partly soluble' is used to describe a mixture where only some of the components dissolve. The term 'miscible' is used to describe a liquid that is miscible in all proportions with the stated solvent.

Abacavir Sulphate. Soluble in *water*.

Abiraterone Acetate. Soluble in *dichloromethane*.

Acacia and Acacia Powder. Almost entirely soluble in twice its weight of *water* yielding a very viscous, slightly acidic solution which is slightly glairy and, when diluted with more *water* and allowed to stand, yields a very small amount of gummy deposit; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Acamprosate Calcium. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *dichloromethane*.

Acarbose. Very soluble in *water*; soluble in *methanol*; practically insoluble in *dichloromethane*.

Acebutolol Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; very slightly soluble in *acetone* and in *dichloromethane*; practically insoluble in *ether*.

Aceclofenac. Freely soluble in *acetone*; soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Acepromazine Maleate. Freely soluble in *dichloromethane*; soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *ether*.

Acesulphame potassium. Soluble in *water*; very slightly soluble in *acetone* and in *ethanol (95 per cent)*.

Glacial Acetic Acid. Miscible with *water*; with *ethanol (95 per cent)*; with *dichloromethane* and with *ether*.

Acetazolamide. Very slightly soluble in *water*; slightly soluble in *ethanol (95 per cent)*. It dissolves in dilute solutions of alkali hydroxides.

Aciclovir. Freely soluble in *dimethylsulphoxide*; slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)*. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

Acitretin. Slightly soluble in *acetone* and in *ethanol (95 per cent)*; very slightly soluble in *cyclohexane*; sparingly soluble in *tetrahydrofuran*; practically insoluble in *water*.

Adefovir Dipivoxil. Freely soluble in *ethanol* and *chloroform*.

Adenosine. Soluble in hot *water*; slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *dichloromethane*.

Adipic Acid. Freely soluble in *ethanol* and in *methanol*; soluble in *acetone* and in boiling *water*; sparingly soluble in *water*.

Adrenaline Tartrate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Adrenaline. Sparingly soluble in *water* ; insoluble in *ethanol (95 per cent)* and in *ether*. It is soluble in solutions of mineral acids, of *sodium hydroxide* and of *potassium hydroxide* but not in solutions of *ammonia* and of the alkali carbonates. It is not stable in a neutral or alkaline which rapidly becomes red on exposure to air.

Agomelatine. Soluble in *methanol*, *dichloromethane* and *chloroform*.

Albendazole. Freely soluble in *formic acid*; sparingly soluble in *dimethylformamide*; slightly soluble in *chloroform*; very slightly soluble in *methanol*. It is slightly soluble in *dilute acids* and insoluble in solutions of alkalis.

Alfuzosin Hydrochloride. Freely soluble in *water*, sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *dichloromethane*.

Alginic Acid. Soluble in alkaline solutions; insoluble but swells in *water*; very slightly soluble in *ethanol (95 per cent)* or practically insoluble in organic solvents.

Allantoin. Slightly soluble in *water*; very slightly soluble in *ethanol*.

Allopurinol. Soluble in dilute solutions of alkali hydroxides; very slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Aloes. Soluble in hot *ethanol (95 per cent)*; powdered Aloes is almost entirely soluble in *ethanol (60 per cent)*; partly soluble in boiling *water*; practically insoluble in *chloroform* and in *ether*.

Alprazolam. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; sparingly soluble in *acetone*; slightly soluble in *ethyl acetate*; insoluble in *water*.

Alprostadil. Freely soluble in *ethanol*; soluble in *acetone*; slightly soluble in *ethyl acetate*; practically insoluble in *water*.

Aluminium Hydroxide, Dried. Dissolves in dilute mineral acids and in excess of caustic alkali solutions. Insoluble in *water* and in *ethanol (95 per cent)*.

Aluminium Magnesium Silicate. Practically insoluble in *water* and in organic solvents. It swells in *water* to produce colloidial dispersion.

Amantadine Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; soluble in *chloroform*; practically insoluble in *ether*.

Ambrisentan. Sparingly soluble in *dimethylformamide*.

Ambroxol Hydrochloride. Soluble in *methanol*; sparingly soluble in *water*; practically insoluble in *dichloromethane*.

Amifostine. Freely soluble in *water*.

Amikacin. Sparingly soluble in *water*.

Amikacin Sulphate. Freely soluble in *water*; practically insoluble in *methanol*, in *acetone*, in *ether* and in *chloroform*.

Amiloride Hydrochloride. Slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Aminocaproic Acid. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *chloroform* and in *ether*. It is soluble in acids and in alkalis.

Aminophylline. Freely soluble in *water* (the solution usually becomes turbid on standing); practically insoluble in *ethanol* and in *ether*.

Amiodarone Hydrochloride. Freely soluble in *dichloromethane*; soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Amisulpride. Freely soluble in *dichloromethane*, sparingly soluble in *ethanol* and practically insoluble in *water*.

Amitraz. Freely soluble in *acetone;* practically insoluble in *water*. Decomposes slowly in *ethanol (95 per cent)*.

Amitriptyline Hydrochloride. Freely soluble in *water*, in *ethanol (95 per cent)*, in *chloroform* and in *methanol*; practically insoluble in *ether*.

Amlodipine Besylate. Freely soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *2-propanol* and in *water*.

S-Amlodipine. Soluble in *methanol*.

Ammonium Chloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Amodiaquine Hydrochloride. Soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Amorolfine Hydrochloride. Soluble in *water* and *ethanol*.

Amoxapine. Freely soluble in *chloroform*; soluble in *tetrahydrofuran*; sparingly soluble in *methanol* and in *toluene*; slightly soluble in *acetone*; practically insoluble in *water*.

Amoxycillin Sodium. Very soluble in *water*; sparingly soluble in *ethanol*; very slightly soluble in *acetone*; practically insoluble in *chloroform* and in *ether*.

Amoxycillin Trihydrate. Slightly soluble in *water*, in *ethanol (95 per cent)* and in *methanol*; soluble in dilute solutions of acids and of alkali hydroxides; practically insoluble in *chloroform*, in *ether* and in fixed oils.

Amphotericin B. Soluble in *dimethylsulphoxide;* slightly soluble in *dimethylformamide* and in *methanol*; insoluble in *benzene*, in *ethanol (95 per cent)*, in *ether* and in *water*.

Ampicillin. Sparingly soluble in *water*; practically insoluble in *ethanol*, in *chloroform*, in *ether* and in fixed oils. It is soluble in dilute solutions of acids and of alkali hydroxides.

Ampicillin Sodium. Freely soluble in *water*; sparingly soluble in *acetone*; slightly soluble in *chloroform*; practically insoluble in *ether*, in *liquid paraffin* and in fixed oils.

Ampicillin Trihydrate. Slightly soluble in *water*; soluble in dilute solution of acids and of alkali hydroxides; practically insoluble in *ethanol (95 per cent)*, in *chloroform*, in *ether* and in fixed oils.

Amprolium Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *ether*; practically insoluble in *dichloromethane*.

Alpha Amylase. Sparingly soluble in *water* (except when admixed with an insoluble diluents); insoluble in *ethanol (95 per cent)* and in *ether*.

Analgin. Very soluble in *water*, soluble in *ethanol (95 per cent)*, practically insoluble in *dichloromethane*.

Anastrozole. Very soluble in *acetonitrile*; freely soluble in *methanol*, in *acetone*, in *ethanol*; and in *tetrahydrofuran*.

Apremilast: Soluble in *dimethyl sulphoxide*; sparingly soluble in *acetonitrile*; slightly soluble in *methanol*; practically insoluble in *water*.

Aprepitant: Sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *acetonitrile*; practically insoluble in *water*.

Aprotinin. Soluble in *water* and in isotonic solutions, practically insoluble in organic solvents.

Arbidol Hydrochloride. Freely soluble in *dimethylsulphoxide*.

Arginine. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; insoluble in *ether*.

Aripiprazole. Freely soluble in *dimethylformamide*; sparingly soluble in *acetone* and insoluble in *water*.

Armodafinil. Freely soluble in *dimethylformamide*; soluble in *methanol*, insoluble in *water*.

Arteether. Soluble in *acetone*, *dichloromethane*, *ethyl acetate*, *hexane* and *methanol*.

Artemether. Very soluble in *dichloromethane* and *acetone*; freely soluble in *ethylacetate* and practically insoluble in *water*.

Arteroiane Maleate. Freely soluble in *methanol*, *ethanol* and slightly soluble in *water*.

Artesunate. Soluble in *ethyl acetate*; practically insoluble in *petroleum ether*.

Ascorbic Acid. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; insoluble in *ether* and in *benzene*.

Ascorbyl Palmitate. Freely soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*, in *dichloromethane* and in fatty oils.

Asenapine Maleate. Soluble in *dichloromethane* and insoluble in *water*.

Aspartame. Sparingly soluble or slightly soluble in *water* and in *ethanol (95 per cent)*, practically insoluble in *n-hexane* and in *dichloromethane*.

Aspirin. Freely soluble in *ethanol (95 per cent)*; soluble in *chloroform* and in *ether*; slightly soluble in *water*.

Atazanavir Sulphate: Sparingly soluble in *methanol* and in *water*.

Atenolol. Soluble in *ethanol*; sparingly soluble in *water*; slightly soluble in *dichloromethane*; practically insoluble in ether.

Atomoxetine Hydrochloride. Freely soluble in *methanol* and in *dichloromethane*; soluble in *water*.

Atorvastatin Calcium. Freely soluble in *methanol*; slightly soluble in *ethanol (95 per cent)* and very slightly soluble in *water*.

Atosiban Acetate. Soluble in *water*.

Atracurium Besylate. Very soluble in *acetonitrile*, in *ethanol* (95 per cent) and in *methylene chloride*; soluble in *water*.

Atropine Methonitrate. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Atropine Sulphate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)* and in *glycerin*; practically insoluble in *chloroform* and in *ether*.

Azacitidine. Soluble in *dimethylsulphoxide*.

Azathioprine. Soluble in dilute solutions of alkali hydroxides; sparingly soluble in dilute mineral acids and practically insoluble in *water* and in *ethanol (95 per cent)*

Azelastine Hydrochloride. Soluble in *ethanol* and in *dichloromethane*; sparingly soluble in *water*.

Azelnidipine. Freely soluble in *ethanol*, in *acetic acid*; practically insoluble in *water*.

Azithromycin. Freely soluble in *anhydrous ethanol* and in *dichloromethane*; practically insoluble in *water*.

Bacitracin. Freely soluble in *water* and in *ethanol (95 per cent)*; soluble in *methanol* and in *glacial acetic acid*; practically insoluble in *acetone*, in *chloroform* and in *ether*.

Bacitracin Zinc. Slightly soluble in *water* and in *ethanol (95 per cent)*; very slightly soluble in *ether*; insoluble in *chloroform*.

Baclofen. Slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

Bambuterol Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*.

Barium Sulphate. Very slightly soluble in acids and in solutions of alkali hydroxides, practically insoluble in *water* and in organic solvents.

Basil Oil (Methyl Chavicol Type). The material shall not require more than seven volumes of *ethanol (70 per cent)* to obtain a clear solution at 27° when tested.

Beclomethasone Dipropionate. Freely soluble in *acetone* and in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Beeswax, White. Soluble in volatile and fixed oils; partially soluble in hot *ethanol (90 per cent)* and in *ether*; practically insoluble in *water*.

Beeswax, Yellow. Soluble in volatile and fixed oils; partially soluble in hot *ethanol (90 per cent)* and in *ether*; practically insoluble in *water*.

Benazepril Hydrochloride. Freely soluble in *ethanol*, slightly soluble in *water*, very slightly soluble in *ethyl acetate* and practically insoluble in *cyclohexane*.

Bendamustine Hydrochloride. Freely soluble in *methanol*, slightly soluble in *water*; practically insoluble in *chloroform*.

Bendrofluazide. Freely soluble in *acetone*, soluble in *ethanol* (95 per cent) and practically insoluble in *water*.

Bentonite. Insoluble in *water* but swells into a homogeneous mass; insoluble in, and does not swell in, organic solvents.

Benzalkonium Chloride. Very soluble in *water* and in *ethanol* (95 per cent).

Benzalkonium Chloride Solution. Miscible with *water* and with *ethanol (95 per cent)*.

Benzathine Penicillin. Freely soluble in *formamide* and *dimethylformamide*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *chloroform* and in *water*; practically insoluble in *ether*.

Benzhexol Hydrochloride. Soluble in *ethanol (95 per cent)*, in *methanol* and in *chloroform*; slightly soluble in *water*.

Benzocaine. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; soluble in dilute acids and very slightly soluble in *water*.

Benzoic Acid. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; soluble in fixed oils, boiling *water* and slightly soluble in *water*.

Hydrous Benzoyl Peroxide. Soluble in *acetone*, in *chloroform* and in *ether*; sparingly soluble in *ethanol* and in *water*.

Benzyl Alcohol. Soluble in *water*, miscible with *ethanol (95 per cent)* and with fatty and essential oils.

Benzyl Benzoate. Soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; practically insoluble in *water*; insoluble in *glycerin*.

Benzylpenicillin Potassium. Very soluble in *water*; practically insoluble in *chloroform*, in ether, in fixed oils and in *liquid paraffin*.

Benzylpenicillin Sodium. Very soluble in *water*; practically insoluble in *chloroform*, in *ether*, in fixed oils and in *liquid paraffin*.

Berberine Chloride. Very slightly soluble in *water*; slightly soluble in *alcohol*, sparingly soluble in *methanol*.

Betahistine Hydrochloride. Very soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *2-propanol*.

Betahistine Mesylate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; very slightly soluble in *2-propanol*.

Betamethasone. Sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *chloroform*; practically insoluble in *water*.

Betamethasone Dipropionate. Freely soluble in *acetone*, in *dichloromethane* and in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Betamethasone Sodium Phosphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform*, in *dichloromethane* and in *ether*.

Betamethasone Valerate. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; practically insoluble in *water* and in *light petroleum*.

Betaxolol Hydrochlroide. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; soluble in *dichloromethane*.

Bezafibrate. Freely soluble in *dimethylformamide*, sparingly soluble in *acetone* and in *ethanol (95 per cent)*. It dissolves in dilute solutions of alkali hydroxides and practically insoluble in *water*.

Biapenem. Sparingly soluble in pH 7.0 buffer and slightly soluble in *water*.

Bicalutamide. Soluble in *dimethylformamide* and sparingly soluble in *methanol*.

Bifonazole. Sparingly soluble in *anhydrous ethanol*; practically insoluble in *water*.

Biotin. Very slightly soluble in *water* and in *ethanol*; insoluble in other common organic solvents.

Biperiden Hydrochloride. Slightly soluble in *water*, and in *ethanol (95 per cent)*; very slightly soluble in *dichloromethane*; practically insoluble in *ether*.

Bisacodyl. Soluble in *acetone*, in *benzene* and in *chloroform*; sparingly soluble in *ethanol (95 per cent)* and in *methanol*; slightly soluble in *ether*; practically insoluble in *water*. It dissolves in dilute mineral acids.

Bismuth Subcarbonate. Practically insoluble in *water*, in *ethanol (95 per cent)* and in *ether*. It is soluble with effervescence in mineral acids.

Bisoprolol Fumarate. Very soluble in *water* and in *methanol*; freely soluble in *chloroform*, in *glacial acetic acid*, and *in alcohol*; slightly soluble in *acetone* and in *ethyl acetate*.

Black Pepper Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Bleomycin Sulphate. Very soluble in *water*.

Boric Acid. Freely soluble in boiling *water*, in boiling *ethanol (95 per cent)* and in *glycerin (85 per cent)*; soluble in *water* and in *ethanol (95 per cent)*.

Bortezomib. Sparingly soluble in *methanol*.

Bosentan Monohydrate. Soluble in *dimethylformamide* and *acetonitrile*.

Bosutinib. Soluble in *acetone*; slightly soluble in *ethyl acetate*; insoluble in *water*.

Brimonidine Tartrate. Soluble in *water*, in *dimethylsulphoxide* and in *methanol*.

Brinzolamide. Slightly soluble in *methanol* and insoluble in *water*.

Brivaracetam. Very soluble in *methanol*; soluble in *water*; practically insoluble in *hexane*.

Bromhexine Hydrochloride. Very slightly soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *methylene chloride*.

Bromocriptine Mesylate. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)*; sparingly soluble in *dichloromethane*; practically insoluble in *water*.

Bronopol. Soluble in *water*, *ethanol (95 per cent)*, *ethyl acetate*; slightly soluble in *chloroform*, *acetone*, *ether* and *benzene*.

Buclizine Hydrochloride. Sparingly soluble in *propane-1,2-diol*; very slightly soluble in *ethanol (95 per cent)* and practically insoluble in *water*.

Budesonide. Freely soluble in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)*.

Bumetanide. Soluble in *acetone* and in *ethanol (95 per cent)*; slightly soluble in *dichloromethane*; practically insoluble in *water*.

Bupivacaine Hydrochloride. Freely soluble in *ethanol (95 per cent)*; soluble in *water*; slightly soluble in *acetone*, in *chloroform* and in *ether*.

Buprenorphine Hydrochloride. Soluble in *methanol*; slightly soluble in *water* and in *chloroform*.

Bupropion Hydrochloride. Soluble in *water*, *0.1M hydrochloric acid* and in *ethanol (95 per cent)*.

Buserelin. Sparingly soluble in *water* and in dilute acids.

Buspirone Hydrochloride. Freely soluble in *methanol* and in *water*; practically insoluble in *acetone*.

Busulphan. Sparingly soluble in *acetone*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Butylated Hydroxytoluene. Very soluble in *acetone*, in *chloroform* and in *ether*; freely soluble in *ethanol (95 per cent)*, in *methanol*, in fixed oils and in fats; insoluble in *water*.

Butylparaben. Freely soluble in *acetone*, in *ethanol (95 per cent)*, in *chloroform* and in *propylene glycol*.

Cabergoline. Freely soluble in *ethanol (95 per cent)*, slightly soluble in *0.1 M hydrochloric acid*, very slightly soluble in *hexane* and practically insoluble in *water*.

Caffeine. Freely soluble in *chloroform* and in boiling *water*; sparingly soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *ether*.

Calamine. practically completely soluble in mineral acids and insoluble in *water*.

Alphacalcidol. Freely soluble in *ethanol (95 per cent)*; soluble in fatty oils; practically insoluble in *water*. It is sensitive to air, heat and light. A reversible isomerisation to pre-alfacalcidol takes place in solution, depending on temperature and time. The activity is due to both compound.

Calcipotriol Anhydrous. Freely soluble in *ethanol (95 per cent)*, slightly soluble in *dichloromethane* and practically insoluble in *water*. It is sensitive to heat and light. A reversible isomerisation to pre-calcipotriol takes place in solution, depending on temperature and time. The activity is due to both compounds.

Calcitriol. Freely soluble in *ethanol*; soluble in fatty oils; practically insoluble in *water*.

Calcium Carbonate. Slightly soluble in *water* containing *carbon dioxide* or any *ammonium salt*; practically insoluble in *water* and in *ethanol (95 per cent)*. It is soluble with effervescence in dilute acids.

Calcium Chloride. Freely soluble in *water* and in *ethanol (95 per cent)*.

Calcium Dobesilate Monohydrate. Very soluble in *water*, freely soluble in *ethanol*, very slightly soluble in *2-propanol* and practically insoluble in *dichloromethane*.

Calcium Folinate. Sparingly soluble in *water*; practically insoluble in *ethanol* and in *ethanol (95 per cent)*.

Calcium Gluconate. Freely soluble in boiling *water*; sparingly soluble in *water*; insoluble in *ethanol (95 per cent)*.

Calcium Lactate. Freely soluble in hot *water*; soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Calcium Levulinate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; insoluble in *ether* and in *chloroform*.

Calcium Pantothenate. Freely soluble in *water*; soluble in *glycerin*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Dibasic Calcium Phosphate. Practically insoluble in *water* and in *ethanol (95 per cent)* It dissolves in *dilute hydrochloric acid* and in *nitric acid*.

Tribasic Calcium Phosphate. Practically insoluble in *water* and in *ethanol (95 per cent)*. It dissolves in *dilute hydrochloric acid* and in *nitric acid*.

Calcium Stearate. Slightly soluble in *ethanol (95 per cent)* and practically insoluble in *water*, in *ether*, in *chloroform*, in *acetone*.

Candesartan Cilexetil. Sparingly soluble in *methanol*; practically insoluble in *water*.

Capecitabine. Freely soluble in *methanol*; soluble in *acetonitrile* and in *alcohol*; sparingly soluble in *water*.

Capreomycin sulphate. Freely soluble in *water*; practically insoluble in most organic solvents.

Captopril. Freely soluble in *water*, in *methanol*, in *ethanol* (95 *per cent*) and in *chloroform*.

Caraway Oil. The material shall not require more than seven volumes of *ethanol (80 percent by volume)* to obtain a clear solution at 27° when tested.

Carbamazepine. Freely soluble in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)* and in *acetone*; practically insoluble in *water* and in *ether*.

Carbenicillin Sodium. Freely soluble in *water*, soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Carbenoxolone Sodium. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Carbidopa. Slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *acetone*, in *chloroform*, in *dichloromethane* and in *ether*. It is soluble in dilute solutions of mineral acids.

Carbimazole. Freely soluble in *chloroform*; soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water* and in *ether*.

Carbomers. Soluble in *water* and in *ethanol (95 per cent)*.

Carboplatin. Sparingly soluble in *water*; very slightly soluble in *acetone* and in *ethanol (95 per cent)*.

Carboprost Trometamol. Soluble in *water*.

Carboxymethylcellulose Calcium. Practically insoluble in *ethanol*, in *acetone*, in *ether*, in *chloroform* and in *benzene*. It swells with *water* to form a suspension.

Carboxymethylcellulose Sodium. Insoluble in *ethanol*, in *ether* and in most organic solvents. Easily dispersed in *water* to form colloidal solution.

Cardamom Oil. The material shall not require more than seven volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 27° when tested.

Carisoprodol. Freely soluble in *ethanol*, in *chloroform*, and in *acetone*; very slightly soluble in *water*.

Carmustine. Freely soluble in *ether*.

Carnauba Wax. Soluble on warming in *chloroform*, in *ethyl acetate* and in *xylene*; practically insoluble in *water* and in *ethanol (95 per cent)*.

Carprofen. Freely soluble in *acetone*; soluble in *methanol*; slightly soluble in *2-propanol*; practically insoluble in *water*.

Carvedilol. Slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water* and in dilute acids.

Castor Oil. Miscible with *chloroform*, with *ethanol (95 per cent)*, with *ether* and with *glacial acetic acid*; slightly soluble in *light petroleum*.

Cefaclor. Slightly soluble in *water*; practically insoluble in *methanol* and in *dichloromethane*.

Cefadroxil. Slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Cefamandole Nafate. Freely soluble in *water*; sparingly soluble in *methanol*.

Cefazolin Sodium. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Cefdinir. Sparingly soluble in *0.1 M phosphate buffer pH 7 solution*; practically insoluble in *water*, in *ethanol*, and in *diethyl ether*.

Cefepime Hydrochloride. Freely soluble in *water* and in *methanol*; practically insoluble in *dichloromethane*.

Cefixime. Soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *ethyl acetate* and in *water*.

Cefoperazone Sodium. Freely soluble in *water*; soluble in *methanol*; slightly soluble in *ethanol (95 per cent)*.

Cefotaxime Sodium. Freely soluble in *water*; practically insoluble in organic solvents.

Cefpirome Sulphate. Freely soluble in *water*.

Cefpodoxime Proxetil. Freely soluble in *dehydrated ethanol*; soluble in *acetonitrile* and in *methanol*; slightly soluble in *ether*; very slightly soluble in *water*.

Ceftazidime. Soluble in *dimethylsulphoxide*; slightly soluble in *dimethylformamide*, in *methanol* and in *water*; insoluble in *acetone*, in *ethanol (95 per cent)*, in *chloroform*, in *dioxan*, in *ethyl acetate*, in *ether* and in *toluene*. It is soluble in alkalis.

Ceftiofur sodium. Freely soluble in *water*.

Ceftriaxone Sodium. Freely soluble in *water*; sparingly soluble in *methanol*; very slightly soluble in *ethanol (95 per cent)*.

Ceftizoxime Sodium. Freely soluble in *water*.

Cefuroxime Axetil. The amorphous form is freely soluble in *acetone*; soluble in *chloroform*, in *ethyl acetate*, and in *methanol*; slightly soluble in *dehydrated alcohol*; insoluble in *ether* and in *water*. The crystalline form is freely soluble in *acetone*; sparingly soluble in *chloroform*, in *ethyl acetate*; and in *methanol*; slightly soluble in dehydrated *alcohol*; insoluble in *ether* and in *water*.

Cefuroxime Sodium. Soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; insoluble in *chloroform*, in *toluene*, in *ether*, in *ethyl acetate* and in *acetone*.

Celecoxib. Freely soluble to soluble in anhydrous *ethanol*; soluble in *dichloromethane* and practically insoluble in *water*.

Celiprolol Hydrochloride. Freely soluble in *water* and *methanol*, soluble in *ethanol (95 per cent)*, very slightly soluble in *dichloromethane*.

Cellulose Acetate Phthalate. Freely soluble in *acetone*; soluble in *diethylene glycol* and in *dioxan*; practically insoluble in *water*, in *ethanol (95 per cent)*, in *toluene* and in chlorinated and non-chlorinated aliphatic hydrocarbons. It dissolves in dilute solutions of alkalis.

Cephalexin. Slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Cephaloridine. Slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Cetirizine Hydrochloride. Freely soluble in *water*; practically insoluble in *acetone* and in *dichloromethane*.

Cetostearyl Alcohol. Freely soluble in *ether*; soluble in *ethanol (95 per cent)* and in *light petroleum*; practically insoluble in *water*. When melted, it is miscible with fixed oils and with *liquid paraffin*.

Cetrimide. Freely soluble in *water*, in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *ether*.

Cetyl Alcohol. Freely soluble in *ether*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*. When melted, it is miscible with *liquid paraffin*, with animal vegetable oils and with melted *wool fat*.

Cetyl Palmitate. Practically insoluble in *water*.

Activated Charcoal. Practically insoluble in all usual solvents.

Chlorambucil. Freely soluble in *ethanol (95 per cent)*, in *acetone* and in *chloroform*; practically insoluble in *water*.

Chloramphenicol. Freely soluble in *ethanol (95 per cent)* and in *1,2-propanediol*; slightly soluble in *water* and in *ether*.

Chloramphenicol Palmitate. Soluble in *acetone*, in *chloroform* and in *ether*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *hexane*; practically insoluble in *water*.

Chloramphenicol Sodium Succinate. Freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Chlorbutol. Freely soluble in *ethanol (95 per cent)*, in *ether*, in *chloroform* and in volatile oils; slightly soluble in *water*.

Chlorcyclizine Hydrochloride. Freely soluble in *water* and in *methylene chloride*, soluble in *alcohol*.

Chlordiazepoxide. Sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *ether*; practically insoluble in *water*.

Chlorhexidine Acetate. Soluble in *ethanol (95 per cent)* and *propylene glycol*.

Chlorhexidine Gluconate Solution. Miscible with *water*; soluble in *ethanol (95 per cent)* and in *acetone*.

Chlorhexidine Hydrochloride. Sparingly soluble in *water* and *propylene glycol*; very slightly soluble in *ethanol (95 per cent)*.

Chlorinated Lime. Partly soluble in *water* and in *ethanol (95 per cent)*.

Chlorocresol. Very soluble in *ethanol (95 per cent)*; freely soluble in *ether* and in fatty oils; slightly soluble in *water*. It dissolves in solutions of alkali hydroxides.

Chloroform. Miscible with *ethanol*, with *ether*, with most organic solvents and with fatty and volatile oils; slightly soluble in *water*.

Chloroquine Phosphate. Freely soluble in *water*; very slightly soluble in *chloroform*, in *ethanol (95 per cent)*, in *ether* and in *methanol*.

Chloroquine Sulphate. Freely soluble in *water* and in *methanol*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Chlorothiazide. Sparingly soluble in *acetone*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Chloroxylenol. Freely soluble in *ethanol (95 per cent)*; soluble in *ether*, in terpenes and in fixed oils; very slightly soluble in *water*. It dissolves in solutions of alkali hydroxides.

Chlorpheniramine Maleate. Freely soluble in *water*; soluble in *ethanol (95 per cent)* and in *chloroform*; slightly soluble in *ether*.

Chlorpromazine Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; soluble in *chloroform*; practically insoluble in *ether*.

Chlorpropamide. Freely soluble in *chloroform* and in *acetone*; soluble in *ethanol (95 per cent)*; slightly soluble in *ether*; practically insoluble in *water*. It dissolves in solutions of alkali hydroxides.

Chlorthalidone. Soluble in *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*, in *ether* and in *chloroform*.

Cholecalciferol. Freely soluble in *ethanol (95 per cent)*, in *acetone*, in *chloroform* and in *ether*; practically insoluble in *water*. It is soluble in fixed oils. Solutions in volatile solvents are unstable and should be used immediately.

Cholecalciferol Concentrate (Powder Form). Practically insoluble, swells or forms a dispersion in *water*, depending on the formulation.

Choline Fenofibrate. Freely soluble in *water*.

Chlortetracycline Hydrochloride. Slightly soluble in *water* and in *ethanol (95 per cent)*. It dissolves in solutions of alkali hydroxide and carbonates.

Chorionic Gonadotrophin. Soluble in *water*; insoluble in *ethanol (95 per cent)*, in *acetone* and in *ether*.

Chymotrypsin. Sparingly soluble in *water*.

Ciclesonide. Very soluble in *dichloromethane*; freely soluble in *methanol* and practically insoluble in *water*.

Cilastatin Sodium. Very soluble in *water* and in *methanol*; slightly soluble in *anhydrous ethanol*; very slightly soluble in *dimethylsulphoxide*; practically insoluble in *acetone* and in *dichloromethane*.

Cilnidipine. Very soluble in *N,N-Dimethyl acetamide*; freely soluble in *acetone*; sparingly soluble in *methanol*; practically insoluble in *water*.

Cilostazol. Freely soluble in *chloroform*; slightly soluble in *methanol* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Cimetidine. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)*; sparingly soluble in *2-propanol*; slightly soluble in *water* and in *chloroform*; practically insoluble in *benzene*, in *dichloromethane* and in *ether*.

Cinacalcet Hydrochloride. Soluble in *dimethylformamide*.

Cinnarizine. Freely soluble in *dichloromethane*; soluble in *acetone*; slightly soluble in *ethanol (95 per cent)*, and in *methanol*; practically insoluble in *water*.

Ciprofloxacin. Very slightly soluble in *ethanol* and in *dichloromethane*; practically insoluble in *water*. It dissolves in *dilute acetic acid*.

Ciprofloxacin Hydrochloride. Soluble in *water*; slightly soluble in *acetic acid* and in *methanol*; very slightly soluble in *ethanol*; practically insoluble in *acetone*, in *acetonitrile*, in *ethyl acetate*, in *hexane* and in *dichloromethane*.

Cisplatin. Sparingly soluble in *dimethylformamide*; slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Citalopram Hydrobromide. Freely soluble in *ethanol* and in *chloroform*.

Citicoline Sodium. Freely soluble in *water* and insoluble in *methanol*.

Citric Acid. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; sparingly soluble in *ether*.

Citric Acid Monohydrate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; sparingly soluble in *ether*.

Clarithromycin. Soluble in *acetone* and *dichloromethane*; slightly soluble in *methanol*; practically insoluble in *water*.

Clemastine Fumarate. Very slightly soluble in *water*; sparingly soluble in *ethanol (70 per cent v/v)*; slightly soluble in *ethanol (50 per cent v/v)* and in *methanol*.

Clindamycin Hydrochloride. Very soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Clindamycin Palmitate Hydrochloride. Very soluble in *water*, slightly soluble in *ethanol*.

Clindamycin Phosphate. Freely soluble in *water*, very slightly soluble in *ethanol (95 per cent)*, practically insoluble in *dichloromethane*.

Clioquinol. Freely soluble in *dimethylformamide* and in *pyridine*; practically insoluble in *water* and in *ethanol (95 per cent)*.

Clobazam. Freely soluble in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)* and slightly soluble in *water*.

Clobetasol Propionate. Freely soluble in *acetone*, sparingly soluble in *ethanol (95 per cent)* and practically insoluble in *water*.

Clobetasone Butyrate. Freely soluble in *acetone* and in *dichloromethane*; slightly soluble in *ethanol (95 per cent)* and practically insoluble in *water*.

Clofazimine. Soluble in *chloroform*, in *dioxan* and in *dimethylformamide;* slightly soluble in *ethanol (95 per cent);* very slightly soluble in *ether;* insoluble in *water*.

Clomifene Citrate. Sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water*; practically insoluble in *ether*.

Clomipramine Hydrochloride. Freely soluble in *water* and *dichloromethane*; soluble in *ethanol (95 per cent)*.

Clonazepam. slightly soluble in *ethanol (95 per cent)*, in *methanol* and practically insoluble in *water*; .

Clonidine Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform*; practically insoluble in *ether*.

Clopidogrel Bisulphate. Freely soluble in *methanol;* practically insoluble in *ether*.

Cloprostenol Sodium. Freely soluble in *water*, in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *acetone*.

Closantel Sodium Dihydrate. Freely soluble in *ethanol (95 per cent)*; soluble in *methanol*; very slightly soluble in *water*.

Clotrimazole. Freely soluble in *acetone*, in *chloroform*, in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Clove Bud Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Clove Leaf Oil. The material shall not require more than seven volumes of *ethanol (82 per cent by volume)* to obtain a clear solution at 27° when tested.

Cloxacillin Benzathine. Freely soluble in *methanol*; soluble in *dichloromethane*; slightly soluble in *water* in *ethanol (95 per cent)* and *2-propanol*.

Cloxacillin Sodium. Freely soluble in *water* and in *methanol*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*.

Clozapine. Freely soluble in *dichloromethane*; soluble in *ethanol*. It dissolves in *dilute acetic acid*; practically insoluble in *water*.

Codeine Phosphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; sparingly soluble in *chloroform*; practically insoluble in *ether*.

Colchicine. Freely soluble in *ethanol (95 per cent)*; soluble in *water*; practically insoluble in *cyclohexane*.

Colistimethate Sodium. Very soluble in *water*, slightly soluble in *ethanol (95 per cent)*, practically insoluble in *acetone*.

Colistin Sulphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*.

Coriander Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Corn Oil. Miscible with *benzene*, *chloroform*, *dichloromethane*, *ether*, *hexane*, and *petroleum ether*; practically insoluble in *ethanol (95 per cent)* and *water*.

Cortisone Acetate. Soluble in *chloroform* and in *dioxan*; sparingly soluble in *acetone*; slightly soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Cottonseed Oil. Slightly soluble in *ethanol (95 per cent)*; miscible with *carbon disulphide*, *chloroform*, *ether*, *hexane*, and *petroleum ether*.

Cresol. Soluble in *water*, usually forming a cloudy solution. Miscible with *ethanol (95 per cent)*, with *chloroform*, with *ether*, with *glycerin* and with fixed and volatile oils.

Cresol with Soap Solution. Miscible up to 10 per cent v/v with *water* and in all proportions with *ethanol (95 per cent)*.

Croscarmellose Sodium. Slightly soluble in *water*; insoluble in *ethanol*, *ether* and in other *organic solvents*.

Crospovidone. Insoluble in *ethanol (95 per cent)*, *ether* and *organic solvents*.

Crotamiton. Slightly soluble in *water*, miscible with *ethanol (95 per cent)*.

Cyanocobalamin. Sparingly soluble in *water* and in *ethanol (95 per cent);* practically insoluble in *chloroform*, in *acetone* and in *ether*.

Cyclizine Hydrochloride. Slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Cyclobenzaprine Hydrochloride. Soluble in *water* and *methanol*.

Alfacyclodextrin. Freely soluble in *water* and in *propylene glycol*, practically insoluble in *ethanol* and in *dichloromethane*.

Betacyclodextrin. Freely soluble in *propylene glycol*; sparingly soluble in *water*; practically insoluble in *ethanol* and in *dichloromethane*.

Cyclopentolate Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*.

Cyclophosphamide. Freely soluble in *ethanol (95 per cent)*; soluble in *water*; slightly soluble in *ether*.

Cycloserine. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Cyclosporine. Soluble in *acetone*, *ethanol (95 per cent)*, *methanol*, *ether*, *chloroform* and in *dichloromethane*; slightly soluble in saturated hydrocarbons; practically insoluble in *water*.

Cyproheptadine Hydrochloride. Freely soluble in *methanol*; soluble in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water*; insoluble in *ether*.

Cyproterone Acetate. Very soluble in *dichloromethane*; freely soluble in *acetone*; soluble in *methanol*; sparingly soluble in *anhydrous ethanol*; practically insoluble in *water*.

Cytarabine. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*.

Dacarbazine. Slightly soluble in *water*, in *ethanol (95 per cent)*; practically insoluble in *dichloromethane*.

Daclatasvir Dihydrochloride. Soluble in *methanol*.

Dalteparin Sodium. Freely soluble in *water*.

Danazol. Freely soluble in *chloroform*; soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)* and in *benzene*; slightly soluble in ether; practically insoluble in *water* and in *hexane*.

Dapoxetine Hydrochloride. Soluble in *methanol*, *acetonitrile*, *dichloromethane* and *water*; sparingly soluble in *isopropyl alcohol*.

Dapsone. Freely soluble in *acetone*; soluble in dilute mineral acids; sparingly soluble in *ethanol (95 per cent)* and very slightly soluble in *water*.

Darifenacin Hydrobromide. Soluble in *dichloromethane* and *methanol*.

Darunavir Ethanolate. Soluble in *methanol*.

Daunorubicin Hydrochloride. Freely soluble in *water* and in *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*.

Decoquinate. Very slightly soluble in *chloroform* and in *ether*; insoluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Deferasirox. Slightly soluble in *acetone*.

Dehydroacetic Acid. Freely soluble in *acetone* and in *benzene*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Dehydroemetine Hydrochloride. Soluble in water; slightly soluble in *ethanol (95 per cent)*.

Deltamethrin. Soluble in *ethanol (95 per cent)* and in *acetone*; insoluble in *water*.

2-Deoxy-D-Glucose. Freely soluble in *water*.

Dequalinium Chloride. Slightly soluble in *water* and in *1,2-propanediol*; soluble in boiling *water*.

Desferrioxamine Mesylate. Freely soluble in *water*; slightly soluble in *methanol*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Desmopressin. Soluble in *water*, in *ethanol (95 per cent)* and in *glacial acetic acid*.

Desogestrel. Very soluble in *methanol*; freely soluble in *ethanol* and in *methylene chloride*; practically insoluble in *water*.

Desoxycortone Acetate. Freely soluble in *chloroform*; soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)* and in *dioxan*; slightly soluble in *1,2-propanediol*; practically insoluble in *water*.

Dexamethasone. Sparingly soluble in *ethanol (95 per cent)*, in *acetone* and in *methanol*; slightly soluble in *chloroform*; very slightly soluble in *ether*; practically insoluble in *water*.

Dexamethasone Sodium Phosphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *dioxan*; practically insoluble in *chloroform* and in *ether*.

Dexchlorpheniramine Maleate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*, in *methanol* and in *dichloromethane*.

Dexlansoprazole. Soluble in *water* and *methanol*.

Dextran 1. Very soluble in *water*; very slightly soluble in *ethanol*.

Dextran 40. Very soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Dextran 70. Very soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Dextrin. Very soluble in boiling *water* forming a mucilaginous solution; slowly soluble in cold *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Dextromethorphan Hydrobromide. Freely soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *water*; practically insoluble in *ether*.

Dextropropoxyphene Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Dextropropoxyphene Napsilate. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Dextrose. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Diacerein. Soluble in *dimethylsulphoxide*; practically insoluble in *water*, in *methanol* and in *chloroform*.

Diazepam. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Diazoxide. Freely soluble in *dimethylformamide*; slightly soluble in *ethanol (95 per cent)* and in alkaline solution; practically insoluble in *water* and in *ether*.

Dichlofenthion. Miscible with *ethanol (95 per cent)* and with *dichloromethane*; immiscible with *water*.

Diclazuril. Sparingly soluble in *dimethylformamide*; practically insoluble in *water*, in *ethanol* and in *methylene chloride*.

Diclofenac Diethylamine. Freely soluble in *ethanol (95 per cent)* and in *methanol*; sparingly soluble in *water*; practically insoluble in *1M sodium hydroxide*.

Diclofenac Potassium. Freely soluble in *methanol*; soluble in *ethanol*; sparingly soluble in *water*; slightly soluble in *acetone*.

Diclofenac Sodium. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)*; sparingly soluble in *water* and in *glacial acetic acid*; practically insoluble in *ether*, in *chloroform* and in *toluene*.

Dichlorophen. Very soluble in *ether*; freely soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Dicloxacillin Sodium. Freely soluble in *water*; soluble in *ethanol (95 per cent)* and in *methanol*.

Dicyclomine Hydrochloride. Freely soluble in *ethanol (95 per cent)* and in *chloroform*; soluble in *water*; practically insoluble in *ether*.

Didanosine. Soluble in *water*.

Dienoestrol. Freely soluble in *ethanol (95 per cent)* and in *acetone*; soluble in *ether*; slightly soluble in *chloroform*; practically insoluble in *water*. It is soluble in dilute solutions of alkali hydroxides.

Dienogest. Slightly soluble in *dichloromethane* and practically insoluble in *water*.

Diethanolamine. Very soluble in *water*.

Diethylcarbamazine Citrate. Very soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform* and in *ether*.

Diethylene Glycol Monoethyl Ether. Soluble in *water*, *acetone* and *ethanol (95 per cent)*; partially soluble with vegetable oils; practically insoluble in mineral oils.

Diethyl Phenyl Acetamide. Practically insoluble in *water*, soluble in *ethanol (95 per cent)*, in *isopropyl alcohol* and in *ether*.

Diethyl Phthalate. Insoluble in *water*; miscible with *ethanol (95 per cent)* and in *ether*.

Diethyltoluamide. Miscible with *ethanol (95 per cent)*, with *chloroform*, with *ether* and with *2-propanol*; practically insoluble in *water* and in *glycerin*.

Digitoxin. Freely soluble in a mixture of equal volumes of *chloroform* and *methanol*; sparingly soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)*, in *methanol* and in *ether*; practically insoluble in *water*.

Digoxin. Freely soluble in *pyridine* and in a mixture of equal volumes of *dichloromethane* and *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Dihydroergocristine Mesylate. Soluble in *water*.

Dihydroergotamine Mesylate. Sparingly soluble in *chloroform* and in *ethanol (95 per cent)*; slightly soluble in *water* and in *ethanol (95 per cent)*.

Dihydrostreptomycin Sulphate. Freely soluble in *water*; practically insoluble in *dichloromethane*, in *methylene*, in *ethanol (95 per cent)* and in *acetone*.

Diiodohydroxyquinoline. Sparingly soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Dill Seed Oil. The material shall not require more than 0.5 volumes of *ethanol (90 percent by volume)* to obtain a clear solution at 27° when tested.

Diloxanide Furoate. Freely soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)* and in *ether*; very slightly soluble in *water*.

Diltiazem Hydrochloride. Freely soluble in *chloroform*, in *methanol*, in *water* and in *formic acid*; sparingly soluble in *ethanol*; practically insoluble in *ether*.

Dimercaprol. Soluble in *water*, in *ethanol (95 per cent)*, in *methanol* and in *benzyl benzoate*. Dissolves in *arachis oil*.

Dimethicone Activated. Miscible with *chloroform*, with *ether*, with *ethyl acetate*, with *methyl ethyl ketone* and with *toluene*; very slightly soluble in *ethanol*, but *silicon dioxide* remains as a residue in the solvents; practically insoluble in *water* and in *methanol*.

Dimetridazole. Freely soluble in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water* and in *ether*.

Dinitolmide. Soluble in *acetone*; slightly soluble in *dichloromethane*, in *ether* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Dinoprostone. Very soluble in *methanol*; freely soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Diphenhydramine Hydrochloride. Very soluble in *water*; freely soluble in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Diphenoxylate Hydrochloride. Freely soluble in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*; practically insoluble in *ether*.

Dipivefrine Hydrochloride. Very soluble in *methanol*, freely soluble in *water*, *ethanol (95 per cent)* and in *dichloromethane*.

Dipyridamole. Freely soluble in *acetone*; soluble in *ethanol*; practically insoluble in *water*. It dissolves in dilute mineral acids.

Disodium Edetate. Soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Disopyramide. Freely soluble in *dichloromethane*; soluble in *ethanol (95 per cent)*; slightly soluble in *water*.

Disopyramide Phosphate. Soluble in *water*, sparingly soluble in *ethanol* and practically insoluble in *dichloromethane*.

Disulfiram. Freely soluble in *dichloromethane*; soluble in *ether*, in *acetone*, in *carbon disulphide* and in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Dithranol. Soluble in *chloroform* and in fixed oils; slightly soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Divalproex Sodium. Very soluble in *chloroform*; freely soluble in *methanol* and in *ethyl ether*; soluble in *acetone* and practically insoluble in *acetonitrile*.

Dobutamine Hydrochloride. Soluble in *methanol*, sparingly soluble in *ethanol (95 per cent)* and *water*.

Docetaxel Trihydrate. Soluble in *methanol*, in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *water*.

Docusate Sodium. Soluble in *carbon tetrachloride*, in *xylene*, in *acetone*, in *ethanol (95 per cent)*; very slightly soluble in *water*.

Dolutegravir Sodium. Very slightly soluble in *methanol* and practically insoluble in *2-propanol* and *acetonitrile*.

Domperidone. Soluble in *dimethylformamide*; slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Domperidone Maleate. Very slightly soluble in *water*; sparingly soluble in *dimethylformamide*; slightly soluble in *methanol* and *ethanol (95 per cent)*.

Donepezil Hydrochloride. Freely soluble in *chloroform*; soluble in *water* and *glacial acetic acid*; silghtly soluble in *ethanol (95 per cent)* and in *acetonitrile*; practically insoluble in *ethyl acetate* and in *n-hexane*.

Dopamine Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; sparingly soluble in *acetone* and in *dichloromethane*.

Dorzolamide Hydrochloride. Soluble in *water*, slightly soluble in *methanol*, very slightly soluble in *anhydrous ethanol*.

Dothiepin Hydrochloride. Freely soluble in *water*, in *ethanol (95 per cent)* and in *dichloromethane*.

Doxapram Hydrochloride. Soluble in *water*, in *ethanol* and in *dichloromethane*.

Doxepin Hydrochloride. Freely soluble in *water*, in *ethanol (95 per cent)* and in *chloroform*.

Doxofylline. Soluble in *water*, in *acetone*, in *ethyl acetate*, in *benzene*, in *chloroform*, in *dioxane* and in hot *methanol* or in hot *ether*; practically insoluble in *ethyl ether*.

Doxorubicin Hydrochloride. Soluble in *water*; slightly soluble in *methanol*; practically insoluble in *chloroform*, in *ether* and in other organic solvents.

Doxycycline Hydrochloride. Freely soluble in *water* and in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*. It is soluble in solutions of alkali hydroxides and carbonates.

Drospirenone. Freely soluble in *dichloromethane*; soluble in *methanol*, in *acetone*; sparingly soluble in *ethanol*, *ethyl acetate* and insoluble in *water* and *hexane*.

Drotaverine Hydrochloride. Freely soluble in *chloroform*, soluble in *ethanol (95 per cent)* and sparingly soluble in *water*.

Duloxetine Hydrochloride. Freely soluble in *methanol*; sparingly soluble in *water*, practically insoluble in *hexane*.

Dutasteride. Soluble in *methanol*.

Dydrogesterone. Soluble in *acetone*; sparingly soluble in *ethanol(95 per cent)*; practically insoluble in *water*.

Ebastine. Very soluble in *dichloromethane*, sparingly soluble in *methanol*; practically insoluble in *water*.

Eberconazole Nitrate. Freely soluble in *methanol*; soluble in *dichloromethane*; very slightly soluble in *isopropanol* and *water*; insoluble in *hexane*.

Econazole Nitrate. Soluble in *methanol*; sparingly soluble in *dichloromethane*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*; practically insoluble in *ether*.

Efavirenz. Freely soluble in *methanol* and in *dichloromethane*.

Eletriptan Hydrobromide. Freely soluble in *dimethylsulphoxide*.

Emtricitabine. Soluble in *water*; sparingly soluble in *methanol*.

Emulsifying Wax. Partly soluble in *ethanol (95 per cent)*; practically insoluble in *water*, forming an emulsion.

Enalapril Maleate. Freely soluble in *methanol* and in *dimethylformamide*; soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; slightly soluble in semipolar organic solvents; practically insoluble in nonpolar organic solvents.

Enoxaparin Sodium. Freely soluble in *water*.

Enrofloxacin. Freely soluble in *methylene chloride*; slightly soluble in *methanol*; practically insoluble in *water*.

Entacapone. Slightly soluble in *ethanol*, *chloroform*, *isopropanol*, and *ether*; very slightly soluble in *toluene*; sparingly soluble in *acetone* and in *methanol*; practically insoluble in *water*.

Entecavir. Soluble in *dimethylsulphoxide*.

Epalrestat. Soluble in *N,N-dimethylformamide*; slightly soluble in *methanol* and in *ethanol*; practically insoluble in *water*.

Ephedrine Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Epinastine Hydrochloride. Freely soluble in *water* and in *methanol*, sparingly soluble in *dichloromethane*, slightly soluble in *acetonitrile*.

Epirubicin Hydrochloride. Soluble in *water* and in *methanol*, slightly soluble in *anhydrous ethanol*, practically insoluble in *acetone*.

Eplerenone. Soluble in *chloroform* and *dichloromethane*; very slightly soluble in *water*.

Eptifibatide. Soluble in 1 per cent v/v *glacial acetic acid* in *water*.

Ergocalciferol. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; practically insoluble in *water*. It is soluble in fixed oils. Solutions in volatile solvents are unstable and should be used immediately.

Ergometrine Maleate. Soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Ergotamine Tartrate. Slightly soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *ether*. It dissolves in *water* but the solution may become hazy which may be prevented by the addition of *tartaric acid*.

Erlotinib hydrochloride. Slightly soluble in *methanol*; practically insoluble in *acetonitrile*, *acetone*, *ethyl acetate* and *hexane*.

Erythromycin. Freely soluble in *ethanol (95 per cent)*; soluble in *chloroform* and in *methanol*; slightly soluble in *water*. It dissolves in *2 M hydrochloric acid*.

Erythromycin Stearate. Soluble in *ethanol*, in *methanol*, in *acetone* and in *chloroform* (solutions in these solvents may be opalescent); practically insoluble in *water*.

Escitalopram Oxalate. Freely soluble in *methanol*, in *dimethylsulphoxide*; sparingly soluble in *water*, in *ethanol*; slightly soluble in *ethyl acetate*; practically insoluble in *heptane*.

Eslicarbazepine Acetate. Sparingly soluble in *methanol*; slightly soluble in *isopropyl alcohol* and practically insoluble in *water*.

Esmolol Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*.

Esomeprazole Magnesium. Soluble in *N,N-dimethylformamide*.

Estradiol Hemihydrate. Soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *methylene chloride*; practically insoluble in *water*.

Ethacrynic Acid. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; very slightly soluble in *water*. It dissolves in ammonia and dilute aqueous solutions of alkali hydroxides and carbonates.

Ethambutol Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; very slightly soluble in *ether*.

Ethanol. Miscible with *water*, with *chloroform*, with *ether* and with *glycerin*.

Ethanol (95 per cent). Miscible in all proportions with *water*, with *chloroform*, with *ether* and with *glycerin*.

Ethanolamine. Miscible with *water* and with *ethanol (95 per cent)*; slightly soluble in *ether*.

Ether Anaesthetic. Soluble in *water*; miscible with *ethanol (95 per cent)*, with *chloroform*, with *benzene* and with fixed and volatile oils.

Ethinyloestradiol. Freely soluble in *ethanol (95 per cent)* and in *ether*; sparingly soluble in *chloroform*; practically insoluble in *water*. It dissolves in dilute solutions of alkalis.

Ethionamide. Soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform* and in *ether*; practically insoluble in *water*.

Ethopabate. Soluble in *dichloromethane* and in *methanol*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *ether*; very slightly soluble in *water*.

Ethopropazine Hydrochloride. Freely soluble in *chloroform* and in *ethanol (95 per cent)*; sparingly soluble in *acetone*; slightly soluble in *water*; practically insoluble in *ether*.

Ethosuximide. Freely soluble in *water*; very soluble in *ethanol (95 percent)* and in *dichloromethane*.

Ethyl Acetate. Soluble in *water*; miscible with *ethanol*, with *ether*, with fixed oils and with volatile oils.

Ethylcellulose. Insoluble in *water*, in *glycerin*, and in *propylene glycol*. *Ethylcellulose* containing less than 46.5 per cent of ethoxy groups is freely soluble in *tetrahydrofuran*, in *methyl acetate*, in *chloroform*, and in mixtures of aromatic hydrocarbons with *ethanol (95 per cent)*. *Ethylcellulose* containing not less than 46.5 per cent of ethoxy groups is freely soluble in *ethanol (95 per cent)*, in *methanol*, in *toluene*, in *chloroform* and in *ethyl acetate*.

Ethyl Chloride. Slightly soluble in *water*; miscible with *ethanol (95 per cent)* and with *ether*.

Ethyl Oleate. Practically insoluble in *water*; miscible with *ethanol (95 per cent)*, with *chloroform*, with *ether* and with *fixed oils*.

Ethyl Paraben. Freely soluble in *ethanol (95 per cent)* and in *methanol*; very slightly soluble in *water*.

Ethyl Vanillin. Freely soluble in *ethanol*, *chloroform*, *ether*, and in solutions of alkali hydroxides; sparingly soluble in *water* at 50°.

Ethylenediamine Hydrate. Slightly soluble in *chloroform* and in *ether*; miscible with *water* and with *ethanol (95 per cent)*.

Ethyloestrenol. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; practically insoluble in *water*.

Ethynodiol Diacetate. Very soluble in *chloroform*; freely soluble in *ether*; soluble in *ethanol*; sparingly soluble in fixed oils; insoluble in *water*.

Etidronate Disodium. Freely soluble in *water*, practically insolnble in *acetone* and in *ethanol (95 per cent)*.

Etizolam. Soluble in *ethanol*, sparingly soluble in *acetonitrile* and in *acetic anhydride* and practically insoluble in *water*.

Etodolac. Soluble in *ethanol (95 per cent)*, in *chloroform*, in *dimethylsulphoxide*, in *polyethylene glycol*; practically insoluble in *water*.

Etoposide. Sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*; practically insoluble in *water*.

Etoricoxib. Freely soluble in *tetrahydrofuran*, *dimethylsulphoxide* and in *dimethylformamide*, soluble in *methanol* and in *acetone*, sparingly soluble in *ethanol*.

Eucalyptus Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Exemestane. Freely soluble in *dichloromethane*, soluble in *methanol*, practically insoluble in *water*.

Ezetimibe. Freely soluble in *methanol*, in *ethanol* and in *acetone*.

Famciclovir. Freely soluble in *methanol* and in *acetone*; sparingly soluble in *ethanol* and in *isopropanol*.

Famotidine. Freely soluble in *dimethylformamide* and in *glacial acetic acid*; slightly soluble in *methanol*; very slightly soluble in *water*; practically insoluble in *acetone*, in *ethanol (95 per cent)*, in *chloroform*, in *ether* and in *ethyl acetate*.

Fasudil Hydrochloride. Freely soluble in *water*; soluble in *dimethylsulfoxide* and insoluble in *ethanol*.

Favipiravir. Sparingly soluble in *acetone* and in *methanol*, slightly soluble in *water*.

Febantel. Soluble in *acetone*; slightly soluble in *anhydrous ethanol*; practically insoluble in *water*.

Felodipine. Freely soluble in *acetone*, in *ethanol*, in *methanol*, and in *dichloromethane*.

Fenbendazole. Sparingly soluble in *dimethylformamide*; very slightly soluble in *methanol*; practically insoluble in *water*.

Fenofibrate. Very soluble in *dichloromethane*, slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Fenspiride Hydrochloride. Soluble in *water*.

Fentanyl. Freely soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Fentanyl Citrate. Freely soluble in *methanol*; soluble in *water*, sparingly soluble in *ethanol (95 per cent)*.

Ferrous Ascorbate. Freely soluble in *water* and insoluble in organic solvents.

Ferrous Fumarate. Slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Ferrous Gluconate. Freely but slowly soluble in *water*; more readily soluble on warming; practically insoluble in *ethanol (95 per cent)*.

Ferrous Sulphate. Very soluble in boiling *water*; freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Ferrous Sulphate Dried. Dissolves slowly, but almost completely, in freshly boiled and cooled *water*.

Fesoterodine Fumarate. Freely soluble in *methanol*, *water* and insoluble in *hexane*.

Fexofenadine Hydrochloride. Freely soluble in *methanol*.

Finasteride. Freely soluble in *ethanol* and in *dichloromethane*; practically insoluble in *water*.

Fingolimod Hydrochloride. Freely soluble in *water* and in *ethanol*; soluble in *propylene glycol*.

Flavoxate Hydrochloride. Sparingly soluble in *dichloromethane*; slightly soluble in *ethanol (95 per cent)* and in *water*.

Flucloxacillin Sodium. Freely soluble in *water* and in *methanol*; soluble in *ethanol (95 per cent)*.

Fluconazole. Slightly soluble in *water*.

Flucytosine. Sparingly soluble in *water*; slightly soluble in *ethanol*; practically insoluble in *ether*.

Fludarabine Phosphate. Freely soluble in *dimethylformamide*; slightly soluble in *water*; very slightly soluble in *ethanol*.

Fludrocortisone Acetate. Sparingly soluble in *ethanol (95 per cent)* and in *chloroform*; slightly soluble in *ether*; practically insoluble in *water*.

Flumazenil. Freely soluble in *dichloromethane*; sparingly soluble in *methanol*; very slightly soluble in *water*.

Flunarizine Dihydrochloride. Sparingly soluble in *methanol* and slightly soluble in *water*, *ethanol* and *dichloromethane*.

Flunixin Meglumine. Freely soluble in *water* and in *methanol*; practically insoluble in *acetone*.

Fluocinolone Acetonide. Soluble in *acetone* and in *ethanol*; slightly soluble in *chloroform* and practically insoluble in *water* and in *light petroleum*.

Fluorescein Sodium. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Fluorometholone. Slightly soluble in *alcohol*; very slightly soluble in *chloroform* and in *ether*; practically insoluble in *water*.

Fluorouracil. Sparingly soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Fluoxetine Hydrochloride. Freely soluble in *methanol*; sparingly soluble in *water* and in *dichloromethane*.

Flupentixol Decanoate. Freely soluble in *ether*; soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Fluphenazine Decanoate. Miscible with *ethanol*, with *chloroform* and with *ether*; practically insoluble in *water*. It dissolves in fixed oils.

Fluphenazine Hydrochloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)* and in *dichloromethane*; practically insoluble in *ether*.

Flurazepam Hydrochloride. Freely soluble in *ethanol*; very soluble in *water*.

Flurbiprofen. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; practically insoluble in *water*. It dissolves in aqueous solutions of alkali hydroxides and carbonates.

Flurbiprofen Sodium. Soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; practically insoluble in *dichloromethane*.

Flutamide. Freely soluble in *acetone* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Fluticasone propionate. Practically insoluble in *water*; sparingly soluble in *dichloromethane*; slightly soluble in *ethanol (95 per cent)*.

Fluvastatin Sodium. Freely soluble in *methanol*; soluble in *water*; practically insoluble in *acetonitrile*.

Fluvoxamine Maleate. Freely soluble in *ethanol (95 per cent)* and in *methanol*; sparingly soluble in *water*.

Folic Acid. Soluble in dilute acids and in alkaline solutions; very slightly soluble in boiling *water*; practically insoluble in cold *water* and in most organic solvents.

Fomepizole. Very soluble in *ethanol*; soluble in *water*.

Formoterol Fumarate Dihydrate. Soluble in *methanol*; slightly soluble in *water*; practically insoluble in *acetonitrile*.

Fosinopril Sodium. Freely soluble in *water*; sparingly soluble in *ethanol*; practically insoluble in *hexane*.

Framycetin Sulphate. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform* and in *ether*.

Frovatriptan Succinate. Slightly soluble in *water*, in *methanol* and insoluble in *methylene dichloride* and in *acetone*.

D-Fructose. Very soluble in *water*; soluble in *ethanol (95 per cent)*.

Frusemide. Soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*. It dissolves in dilute aqueous solutions of alkali hydroxides.

Fulvestrant. Freely soluble in *ethanol (95 per cent)* and *dichloromethane*; practically insoluble in *water*.

Fumaric Acid. Soluble in *ethanol (95 per cent)*; slightly soluble in *water* and in *ether*; very slightly soluble in *chloroform*.

Furazolidone. Slightly soluble in *chloroform*; very slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Fusidic Acid. Freely soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *ether*; practically insoluble in *water*.

Galantamine Hydrobromide. Soluble in *0.1M sodium hydroxide*; sparingly soluble in *water*; very slightly soluble in *ethanol*; insoluble in *n-propanol*.

Gallamine Triethiodide. Very soluble in *water*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *chloroform*; practically insoluble in *ether*.

Ganciclovir. Slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)*. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

Gefitinib. Soluble in *dimethylsulphoxide*.

Gelatin. Soluble in hot *water*; practically insoluble in *water* but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight and in most organic solvents.

Gemcitabine Hydrochloride. Soluble in *water*; slightly soluble in *methanol*; practically insoluble in *acetone*.

Gemfibrozil. Very soluble in *dichloromethane*; freely soluble in *ethanol* and in *methanol*; practically insoluble in *water*.

Gemifloxacin Mesylate. Sparingly soluble in *water*; very slightly soluble in *acetonitrile*; practically insoluble in *methanol*.

Gentamicin Sulphate. Freely soluble in *water*; practically insoluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*.

Glibenclamide. Sparingly soluble in *dichloromethane*; slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water* and *ether*.

Gliclazide. Freely soluble in *dichloromethane*; sparingly soluble in *acetone*; slightly soluble in *ethanol*.

Glimepiride. Soluble in *dimethylformamide*; sparingly soluble in *dichloromethane*; slightly soluble in *methanol*; practically insoluble in *water*.

Glipizide. Very slightly soluble in *dichloromethane* and *acetone*; practically insoluble in *ethanol (95 per cent)* and in *water*.

Glucosamine Sulphate Sodium Chloride. Freely soluble in *water*; sparingly soluble in *methanol*; practically insoluble in *acetone*.

Glutamic Acid. Freely soluble in *water*; practically insoluble in *ethanol* and in *ether*.

Glycerin. Miscible with *water* and with *ethanol (95 per cent)*; slightly soluble in *acetone*; practically insoluble in *ether* and in fixed oils and volatile oils.

Glyceryl Monostearate. Freely soluble in *chloroform*; soluble in *ether*, in *benzene* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Glyceryl Trinitrate Solution, Concentrated. Miscible with *acetone* and with *ether*.

Glycine. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Glycopyrrolate. Soluble in *water* and *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Gramicidin. Soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Granisetron. Soluble to freely soluble in *methylene chloride* and in *methanol*.

Granisetron Hydrochloride. Freely soluble in *water*, sparingly soluble in *methylene chloride*, slightly soluble in *methanol*.

Griseofulvin. Freely soluble in *dimethylformamide* and in *1,1,2,2-tetrachloroethane*; soluble in *acetone* and in *chloroform*; slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Guaiphenesin. Soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *water*; slightly soluble in *ether*.

Guar Gum. When stirred with 50 parts of *water*, a thick jelly is formed which, with further addition of 150 parts of *water*, yields a thick transparent suspension; practically insoluble in *ethanol (95 per cent)*.

Guggul Resin. Sparingly soluble in *ethanol (95 per cent)*, *in methanol* and in *chloroform*; practically insoluble in *water*.

Gugulipid. Soluble in *ethanol (95 per cent)*, in *methanol*, in *ethyl acetate*, and in *chloroform*; insoluble in *water*.

Haloperidol. Soluble in *chloroform*; sparingly soluble in *ethanol (95 per cent)* and in *dichloromethane*; slightly soluble in *ether*; practically insoluble in *water*.

Haloxon. Freely soluble in *ethanol (95 per cent)*, in *dichloromethane* and in *acetone*; practically insoluble in *water*.

Heparin Sodium. Soluble in *water* and in *saline solution* forming a clear, colourless or straw-coloured liquid.

Histamine Phosphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Homatropine Hydrobromide. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *ether*.

Homatropine Methylbromide. Freely soluble in *water* and in *dilute ethanol (95 per cent)*; slightly soluble in *absolute ethanol (95 per cent)*; practically insoluble in *ether*.

Hyaluronidase. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *acetone* and in *ether*.

Hydralazine Hydrochloride. Soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *chloroform*, in *dichloromethane* and in *ether*.

Hydrochloric Acid. Miscible with *water*.

Hydrochlorothiazide. Soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Hydrocortisone. Sparingly soluble in *ethanol (95 per cent)* and in *acetone*; slightly soluble in *chloroform*; very slightly soluble in *ether*; practically insoluble in *water*.

Hydrocortisone Acetate. Slightly soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *water*.

Hydrocortisone Hemisuccinate. Freely soluble in *acetone* and in *ethanol (95 per cent)*; practically insoluble in *ether* and in *water*. It dissolves in solutions of alkali carbonates and alkali hydroxides.

Hydrogenated Castor Oil. Soluble in *acetone*, in *chloroform* and in *carbon tetrachloride*; insoluble in *water*.

Hydrogenated Vegetable Oil. Soluble in *hexane* and in hot *propane-2-ol*; practically insoluble in *water*.

Hydroxocobalamin. Soluble in *water*.

Hydroxychloroquine Sulphate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Hydroxyethylcellulose. Soluble in hot and cold *water* giving a colloidal solution, practically insoluble in *acetone* and in most organic solvents.

Hydroxyprogesterone Hexanoate. Very soluble in *chloroform*; freely soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*. It dissolves in fixed oils and esters.

Hydroxypropylcellulose. Soluble in cold *water*, in *ethanol*, in *methanol* and in *propylene glycol* forming colloidal solutions; slightly soluble in *acetone*; very slightly soluble in *toluene*; practically insoluble in hot *water*.

Hydroxypropylmethylcellulose. Practically insoluble in hot *water*; in *acetone*, in *ethanol*, in *ether* and in *toluene*. It swells in *water* forming an opalescent, viscous colloidal solution.

Hydroxypropyl Methylcellulose Phthalate. Very slightly soluble in *acetone* and in *toluene*; practically insoluble in *water*, in *dehydrated alcohol* and in *hexane*.

Hydroxyurea. Freely soluble in *water* and in hot *ethanol (95 per cent)*.

Hydroxyzine Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; very slightly soluble in *acetone*.

Hyoscine Butylbromide. Freely soluble in *water* and in *chloroform*; sparingly soluble in *ethanol*.

Hyoscine Hydrobromide. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Hyoscyamine Sulphate. Very soluble in *water*; sparingly soluble or soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Ibudilast. Soluble in *methanol*.

Ibuprofen. Freely soluble in *acetone*, in *chloroform*, in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*. It dissolves in dilute solutions of alkali hydroxides and carbonates.

Idoxuridine. Slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*. It dissolves in dilute solutions of alkali hydroxides.

Ifosfamide. Freely soluble in *dichloromethane*; soluble in *water*.

Iloperidone. Freely soluble in *chloroform*; sparingly soluble in *acetone* and insoluble in *water*.

Imatinib Mesylate. Freely soluble in *water*; slightly soluble in *ethanol*, practically insoluble in *methylene chloride*.

Imidurea. Soluble in *water* and in *glycerine*; sparingly soluble in *propylene glycol*; insoluble in most organic solvents.

Imipenem. Sparingly soluble in *water*; slightly soluble in *methanol*.

Imipramine Hydrochloride. Freely soluble in *water*, in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *ether*.

Indapamide. Soluble in *ethanol (95 per cent)*; very slightly soluble in *ether*; practically insoluble in *water*.

Indinavir Sulphate. Freely soluble in *water*; soluble in *methanol*.

Indomethacin. Soluble in *chloroform*; sparingly soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Inositol. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Insulin. Practically insoluble in *water* and in *ethanol (95 per cent)* and in *ether*. It dissolves in dilute mineral acids and, with decomposition, in dilute solutions of alkali hydroxides.

Insulin Aspart. Practically insoluble in *ethanol (95 per cent)*, in *methanol* and in aqueous solutions with a pH around 5.1. In aqueous solutions below pH 3.5 or above pH 6.5, the solubility is greater than or equal to 25 mg per ml.

Insulin Glargine. Soluble in dilute mineral acids; practically insoluble in *water* and in *anhydrous ethanol*.

Insulin, Human. Practically insoluble in *water* and in *ethanol (95 per cent)* and in *ether*. It dissolves in dilute mineral acids and, with decomposition, in dilute solutions of alkali hydroxides.

Insulin Lispro. Practically insoluble in *water* and in *ethanol (95 percent)*. It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

Iodine. Very soluble in concentrated solutions of iodides; soluble in *chloroform* and in *ethanol (95 per cent)*; slightly soluble in *glycerin*; very slightly soluble in *water*.

Iopanoic Acid. Soluble in *ethanol* and in *methanol*; practically insoluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Ipratropium Bromide. Freely soluble in *methanol*; soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Irbesartan Hydrochloride. Sparingly soluble in *methanol*; slightly soluble in *dichloromethane*; practically insoluble in *water*.

Irinotecan Hydrochloride Trihydrate. Freely soluble in *glacial acetic acid*; slightly soluble in *water* and *ethanol (95 per cent)*.

Iron and Ammonium Citrate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Isoflupredone Acetate. Freely soluble in *dimethylsulphoxide*; sparingly soluble in *ethanol (95 per cent)* and in *acetone*; practically insoluble in *water*; in *dichloromethane* and in *ether*.

Isoflurane. Soluble in *ethanol (95 per cent)* and *trichloroethylene*; practically insoluble in *water*.

Isoniazid. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; very slightly soluble in *ether*.

Isoprenaline Hydrochloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; insoluble in *chloroform* and in *ether*.

Isoprenaline Sulphate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Isopropyl Alcohol. Miscible with *water*, *chloroform*, *ether* and *ethanol (95 per cent)*.

Isopropyl Myristate. Immiscible with *water*; miscible *with ethanol (95 per cent)*, with *dichloromethane*, with fatty oils and with *liquid paraffin*.

Isopropyl Palmitate. Miscible with *ethanol (95 per cent)*, with *dichloromethane*, with fatty oils and with *liquid paraffin*; immiscible with *water*.

Isotretinoin. Soluble in *dichloromethane*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Isoxsuprine Hydrochloride. Slightly soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Itopride Hydrochloride. Very soluble in *water*; freely soluble in *methanol*.

Itraconazole. Freely soluble in *dichloromethane*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Ivermectin. Freely soluble in *dichloromethane*; soluble in *ethanol*; practically insoluble in *water*.

Kanamycin Acid Sulphate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *acetone*, in *chloroform* and in *ether*.

Kanamycin Sulphate. Freely soluble in *water*; practically insoluble in *acetone*, in *chloroform*, in *ether* and in *ethanol (95 per cent)*.

Heavy Kaolin. Practically insoluble in *water* and in organic solvents. It does not dissolve in mineral acids and in solutions of alkali hydroxides.

Light Kaolin. Practically insoluble in *water* and in mineral acids.

Ketamine Hydrochloride. Freely soluble in *water* and in *methanol*; soluble in *ethanol (95 per cent)*; sparingly soluble in *chloroform*; practically insoluble in *ether* and in *benzene*.

Ketoconazole. Freely soluble in *dichloromethane*; soluble in *chloroform* and in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water* and in *ether*.

Ketoprofen. Freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*; practically insoluble in *water*.

Ketorolac Tromethamine. Freely soluble in *water* and in *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *dichloromethane*.

Ketotifen Fumarate. Sparingly soluble in *water*; slightly soluble in *methanol*; very slightly soluble in *acetonitrile*.

Labetalol Hydorchloride. Sparingly soluble in *water* and in *ethanol (96 per cent)*; practically insoluble in *methylene chloride*.

Lacidipine. Freely soluble in *acetone*; sparingly soluble in *ethanol*; practically insoluble in *water*.

Lactic acid. Miscible in all proportions with *water*, with *ethanol (95 per cent)* and with *ether*.

Lactose. Freely but slowly soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Lactulose. Freely soluble in *water*; sparingly soluble in *methanol*; practically insoluble in *toluene*.

Lamivudine. Soluble in *water*; sparingly soluble in *methanol*.

Lamotrigine. Slightly soluble in *anhydrous ethanol*; very slightly soluble in *water*; insoluble in *chloroform*.

Lansoprazole. Freely soluble in *dimethylformamide*; practically insoluble in *water*.

Lapatinib Ditosylate. Freely soluble in *dimethylformamide* and insoluble in *water*.

Latanoprost. Very soluble in *acetonitrile*; freely soluble in *acetone*, in *ethanol*, in *ethyl acetate*, in *isopropanol*, in *methanol* and in *octanol*; practically insoluble in *water*.

Lavender Oil. The material shall not require more than seven volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 27° when tested.

Lecithin. Sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water*.

Leflunomide. Freely soluble in *methanol*; sparingly soluble in *dichloromethane*; practically insoluble in *water*.

Lemon Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Lemon Grass Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Lenvatinib Mesylate. Very slightly soluble in *water*; practically insoluble in *ethanol*.

Letrozole. Freely soluble in *methylene chloride*; sparingly soluble in *methanol*; practically insoluble in *water*.

Levamisole Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; slightly soluble in *dichloromethane*; practically insoluble in *ether*.

Levetiracetam. Very soluble in *water*; soluble in *acetonitrile*; practically insoluble in *hexane*.

Levocarnitine. Freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *acetone*; in *ether*; and in *benzene*.

Levocetirizine Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)* and in *methanol*. It dissolves in dilute solutions of alkali hydroxides.

Levodopa. Freely soluble in *1 M hydrochloric acid*; sparingly soluble in *0.1 M hydrochloric acid*; slightly soluble in *water*; practically insoluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*.

Levodropropizine. Freely soluble in *dilute acetic acid*, in *methanol*; slightly soluble in *ethanol (95 per cent)* and in *water*.

Levofloxacin Hemihydrate. Soluble in *dilute sodium hydroxide solution*; sparingly soluble in *acetic acid* and *chloroform*; slightly soluble in *methanol*.

Levonorgestrel. Sparingly soluble in *chloroform* and in *dichloromethane*; slightly soluble in *acetone*, in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Levosalbutamol Hydrochloride. Soluble in *water* and *methanol*.

Levosalbutamol Sulphate. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)* and *dichloromethane*.

Lignocaine. Very soluble in *ethanol* and in *chloroform*; freely soluble in *benzene* and in *ether*; practically insoluble in *water*. Dissolves in oils.

Lignocaine Hydrochloride. Very soluble in *water*; freely soluble in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Lime Oil. The material shall not require more than seven volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 200° when tested.

Lincomycin Hydrochloride. Freely soluble in *water*; soluble in *dimethylformamide*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *acetone*; practically insoluble in *chloroform* and in *ether*.

Linezolid. Soluble in *chloroform* and slightly soluble in *methanol*.

Lindane. Freely soluble in *acetone* and in *ether*; soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Alpha Lipoic Acid. Soluble in *ethanol*; very slightly soluble in *water*.

Lisinopril. Soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Lithium Antimony Thiomalate. Soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *ether*.

Lithium Carbonate. Slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Lomustine. Freely soluble in *acetone*, in *chloroform* and in *dichloromethane*; soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Loperamide Hydrochloride. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)*; slightly soluble in *water*.

Lopinavir. Freely soluble in *methanol* and in *dichloromethane*; practically insoluble in *water*.

Loratadine. Freely soluble in *acetone* and in *methanol*; practically insoluble in *water*.

Lorazepam. Sparingly soluble in *ethanol (95 per cent)* and in *dichloromethane*, practically insoluble in *water*.

Losartan Potassium. Freely soluble in *water*; sparingly soluble in *isopropyl alcohol*; slightly soluble in *acetonitrile*.

Lovastatin. Freely soluble in *chloroform*; soluble in *acetone*, in *acetonitrile* and in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *hexane*; insoluble in *water*.

Lubiprostone. Soluble in *acetone* and *dimethylformamide*; sparingly soluble in aquous buffer.

Luliconazole. Freely soluble in *dimethylformamide* and *acetone*, soluble in *acetonitrile* and *methanol*, sparingly soluble in *ethanol*, and practically insoluble in *water*.

Lumefantrine. Freely soluble in *N,N-dimethylformamide*, *chloroform* and *ethyl acetate*; soluble in *dichloromethane*; slightly soluble in *ethanol* and *methanol*; practically insoluble in *water*.

Lutein. Soluble in *ethanol*, in *ethyl acetate*, in *dichloromethane*; partially soluble in *hexane*.

Lynoestrenol. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*, in *acetone*, and in *ether*; practically insoluble in *water*.

Lysine Hydrochloride. Freely soluble in *water*.

Magaldrate. Soluble in dilute solutions of mineral acids; insoluble in *water* and in *ethanol (95 per cent)*.

Heavy Magnesium Carbonate. Practically insoluble *water*. It dissolves in dilute acids with strong effervescence.

Light Magnesium Carbonate. Practically insoluble in *water*. It dissolves in dilute acids with strong effervescence.

Magnesium Chloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*.

Magnesium hydroxide. Practically insoluble in *water* and in *ethanol (95 per cent)*. It dissolves in dilute acids.

Magnesium Hypophosphite. Very soluble in boiling *water*; freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Heavy Magnesium Oxide. Practically insoluble in *water*; insoluble in *ethanol (95 per cent)*. It dissolves in dilute acids with at most slight effervescence.

Light Magnesium Oxide. Practically insoluble in *water*; insoluble in *ethanol (95 per cent)*. It dissolves in dilute acids with at most slight effervescence.

Magnesium Stearate. Practically insoluble in *water*, in *ethanol* and in *ether*.

Magnesium Sulphate. Very soluble in boiling *water*; freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Magnesium Trisilicate. Practically insoluble in *water* and in *ethanol (95 per cent)*.

Maleic Acid. Freely soluble in *water* and in *ethanol (95 per cent)*.

Malic Acid. Freely soluble in *water* and in *ethanol (95 per cent)*, sparingly soluble in *acetone*.

Malt Extract. Almost completely soluble in cold *water*, more readily in warm *water*. An aqueous solution is not clear and deposits a voluminous precipitate on standing.

Maltitol. Very soluble in *water*; practically insoluble in *anhydrous ethanol*.

Liquid Maltitol. Miscible with *water* and with *glycerol*.

Maltodextrin. Freely soluble in *water*.

Mannitol. Freely soluble in *water*; slightly soluble in *pyridine*; very slightly soluble in *ethanol (95 per cent)*; insoluble in *chloroform* and in *ether*.

Marbofloxacin. Sparingly or slightly soluble in *methylene chloride*; slightly soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Mebendazole. Freely soluble in *formic acid*; practically insoluble in *water*, in *dichloromethane*, in *ethanol (95 per cent)* and in *ether*.

Mebeverine Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Meclizine Hydrochloride. Soluble in *dichloromethane*, in *chloroform* and in *ethanol (95 per cent)*; slightly soluble in *water*; practically insoluble in *ether*.

Meclofenamic Acid. Soluble in *dimethylformamide* and in *1 M sodium hydroxide*; sparingly soluble in *ether*; slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*; practically insoluble in *water*.

Medroxyprogesterone Acetate. Freely soluble in *chloroform*; soluble in *acetone* and in *dioxan*; sparingly soluble in *ethanol (95 per cent)* and in *methanol*; slightly soluble in *ether* and insoluble in *water*.

Mefenamic Acid. Sparingly soluble in *ether*; slightly soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *water*.

Mefloquine Hydrochloride. Soluble in *ethanol (95 per cent)* and in *ethyl acetate*; slightly soluble in *water*.

Megestrol Acetate. Very soluble in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *ether* and in fixed oils; practically insoluble in *water*.

Meloxicam. Soluble in *dimethylformamide*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Melphalan. Slightly soluble in *methanol* and in *ethanol (95 per cent)*; practically insoluble in *water*, in *chloroform* and in *ether*. It dissolves in dilute mineral acids.

Memantine Hydrochloride. Slightly soluble in *water*.

Mentha Oil. 1.0 ml dissolves in 3.5 to 4 ml of *ethanol (70 per cent)*; on further addition of 5 to 10 ml of *ethanol (70 per cent)*, the solution remains clear or is not more then slightly opalescent.

Mentha Arvensis Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Menthol. Very soluble in *ethanol (95 per cent)* and in *ether*; freely soluble in *light liquid paraffin*, in *glacial acetic acid* and in volatile oils; slightly soluble in *water*.

Mephentermine Sulphate. Soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Mepyramine Maleate. Very soluble in *water*; freely soluble in *chloroform* and in ethanol (*95 per cent)*; very slightly soluble in ether.

Mercaptopurine. Slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water* and in *ether*. It dissolves in solutions of alkali hydroxides.

Meropenem. Soluble in *dimethylformamide* and in 5 per cent w/v solution of *monobasic potassium phosphate*; sparingly

soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone* and in *ether*.

Mesalazine. Very slightly soluble in *water*; practically insoluble in *ethanol (95 per cent)*. It dissolves in dilute solutions of alkali hydroxides and in *dilute hydrochloric acid*.

Mesna. Freely soluble in *water*, soluble in *ethanol (95 per cent)*, practically insoluble in *cyclohexane*.

Mestranol. Freely soluble in *chloroform*; soluble in *ether*; slightly soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Metadoxine. Freely soluble in *water*; soluble in *methanol* and *ethanol (95 per cent)*; practically insoluble in *chloroform* and *ether*.

Metformin Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform*, in *dichloromethane* and in *ether*.

Methadone Hydrochloride. Freely soluble in *ethanol (95 per cent)* and in *chloroform*; soluble in *water*; practically insoluble in *ether*.

Methocarbamol. Soluble in *ethanol* only with heating, sparingly soluble in *water* and in *chloroform*; insoluble in *benzene* and in *n-hexane*.

Methotrexate. Practically insoluble in *water*, *1,2-dichloroethane*, in *ethanol (95 per cent)* and in *ether*. It dissolves in dilute solutions of mineral acids and in dilute solutions of alkali hydroxides and in carbonates.

Methoxamine Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; very slightly soluble in *chloroform* and in *ether*.

Methylcellulose. Practically insoluble in hot *water*; in *acetone*, in *ethanol*, in *ether* and in *toluene*. It dissolves in cold *water* forming a colloidal solution.

Methylcobalamin. Sparingly soluble in *water*, slightly soluble in *ethanol* and practically insoluble in *acetonitrile*.

Methyldopa. Slightly soluble in *water*, very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*. It is freely soluble in *dilute hydrochloride acid*.

Methylergometrine Maleate. Slightly soluble in *ethanol (95 per cent)*, slightly soluble in *water*; very slightly soluble in *chloroform* and in *ether*.

Methylparaben. Freely soluble in *ethanol (95 per cent)*, in *ether* and in *methanol*; very slightly soluble in *water*.

Methylphenidate Hydrochloride. Freely soluble in *water* and in *methanol*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform* and in *acetone*.

Methyl prednisolone. Sparingly soluble in *ethanol*; slightly soluble in *acetone* and in *chloroform*; very slightly soluble in *ether*; practically insoluble in *water*.

Methyl Prednisolone Acetate. Slightly soluble in *ethanol*; very slightly soluble in *ether*; practically insoluble in *water*.

Methyl Salicylate. Miscible with *chloroform*, with *ethanol (95 per cent)* and with fixed and volatile oils; very slightly soluble in *water*.

Metoclopramide Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; sparingly soluble in *dichloromethane*; practically insoluble in *ether*.

Metolazone. Sparingly soluble in *methanol*, slightly soluble in *ethyl acetate*, very slightly soluble in *water* and in *dichloromethane*.

Metoprolol Succinate. Freely soluble in *water*; soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *isopropyl alcohol*.

Metoprolol Tartrate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*, in *chloroform* and in *dichloromethane*; slightly soluble in *acetone*; practically insoluble in *ether*.

Metronidazole. Slightly soluble in *water*, in *ethanol (95 per cent)*, in *acetone* and in *dichloromethane*; very slightly soluble in *ether*.

Metronidazole Benzoate. Freely soluble in *chloroform*; in *dichloromethane*; soluble in *acetone*; slightly soluble in *ether* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Mexiletine Hydrochloride. Freely soluble in *water* and in *methanol*; sparingly soluble in *dichloromethane*; practically insoluble in *ether*.

Mianserin Hydrochloride. Soluble in *chloroform* and in *dichloromethane*; sparingly soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Miconazole. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Miconazole Nitrate. Sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)* and in *chloroform*; very slightly soluble in *water* and in *ether*.

Microcrystalline Cellulose. Practically insoluble in *water*, in *acetone*, in *ethanol*, in *toluene*, in dilute acids and in a 5.0 per cent w/v solution of *sodium hydroxide*.

Microcrystalline Wax. Soluble in *chloroform*, in *ether*, in volatile oils and in most warm fixed oils; sparingly soluble in *ethanol*; practically insoluble in *water*.

Midazolam. Freely soluble in *acetone* and in *ethanol (95 per cent)*, soluble in *methanol* and practically insoluble in *water*.

Mifepristone. Freely soluble in *dichloromethane* and in *methanol*; insoluble in *water*.

Minoxidil. Soluble in *methanol* and in *propylene glycol*; slightly soluble in *water*; very slightly soluble in *ether*; practically insoluble in *chloroform* and in *acetone*.

Mirtazapine. Freely soluble in *ethanol*; practically insoluble in *water*.

Misoprostol. Soluble in *water* and *ethanol (95 per cent)*; sparingly soluble in *acetonitrile*.

Mitiglinide Calcium Dihydrate. Soluble in *methanol*, in *acetic acid* and insoluble in *water*.

Mitomycin. Freely soluble in *dimethylacetamide*, slightly soluble in *acetone* and in *water*; sparingly soluble in *methanol*.

Modafinil. Sparingly soluble in *methanol*, slightly soluble in *absolute alcohol*, very slightly soluble in *water*.

Moexipril Hydrochloride. Soluble in *water*.

Mometasone Furoate. Soluble in *acetone* and in *dichloromethane*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Monosulfiram. Freely soluble in organic solvents; practically insoluble in *water*; in acids and in alkalis.

Monothioglycerol. Freely soluble in *water*; miscible with *ethanol (95 per cent)*; practically insoluble in *ether*.

Montelukast Sodium. Freely soluble in *ethanol (95 per cent)*, in *methanol* and in *water*; practically insoluble in *acetonitrile*.

Morphine Sulphate. Freely soluble in hot *water*; soluble in *water*; slightly soluble in *ethanol (95 per cent)* but more soluble in hot *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Mosapride Citrate Dihydrate. Soluble in *dimethylformamide*; sparingly soluble in *methanol*; practically insoluble in *water*.

Moxidectin. Very soluble in *ethanol (95 per cent)*; slightly soluble in *hexane* ; practically insoluble in *water*.

Moxifloxacin Hydrochloride. Sparingly soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*.

Mupirocin. Freely soluble in *acetone*, in *anhydrous ethanol*; slightly soluble in *water* and in *dichloromethane*.

Mustine Hydrochloride. Very soluble in *water*.

Mycophenolate Mofetil. Freely soluble in *acetone*; sparingly soluble in *anhydrous ethanol*; practically insoluble in *water*.

Myristic Acid. Insoluble in *water*.

Nabumetone. Freely soluble in *acetone*, slightly soluble in *methanol*; practically insoluble in *water*.

Nadifloxacin. Soluble in *dimethylformamide*.

Nalidixic Acid. Soluble in *dichloromethane*; slightly soluble in *acetone* and in *ethanol (95 per cent)*; very slightly soluble in *ether*; practically insoluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Nalorphine Hydrochloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*. It dissolves in dilute solution of alkali hydroxides.

Naloxone Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; practically insoluble in *toluene*.

Naltrexone Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *dichloromethane*.

Nandrolone Decanoate. Freely soluble in *chloroform*, in *ethanol (95 per cent)*, in *methanol*, in *ether*, in fixed oils and in esters; practically insoluble in *water*.

Nandrolone Laurate. Freely soluble in *ethanol (95 per cent)*; in *dichloromethane*, in *ether* and in fixed oils and esters of fatty acids; practically insoluble in *water*.

Nandrolone Phenylpropionate. Soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Naphazoline Nitrate. Soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; very slightly soluble in *chloroform*; practically insoluble in *ether*.

Naproxcinod. Soluble in *methanol*.

Naproxen. Soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Natamycin. Very soluble in *ethanol (95 per cent)*, in *ether* and in *ester*; sparingly soluble in *water*.

Nebivolol Hydrochloride. Sparingly soluble in *dimethylformamide*; slighly soluble in *methanol*.

Nelfinavir Mesylate. Soluble in *methanol*.

Neomycin Sulphate. Freely soluble in *water*; in slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform* and in *ether*.

Neostigmine Bromide. Very soluble in *water*; freely soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *ether*.

Neostigmine Methylsulphate. Very soluble in *water*, freely soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *ether*.

Neotame. Very soluble in *ethanol (95 per cent)* and polar organic solvents; sparingly soluble in *water*.

Netilmicin Sulphate. Very soluble in *water*; practically insoluble in *acetone* and in *ethanol (95 per cent)*.

Nevirapine. Soluble in *dimethylsulphoxide*; sparingly soluble in *dichloromethane* and in *dimethylformamide*.

Nicardipine Hydrochloride. Soluble in *dimethylsulphoxide*, slightly soluble in *water* and *methanol*.

Niclosamide. Sparingly soluble in *acetone;* slightly soluble in *ethanol*; practically insoluble in *water.*

Nicorandil. Freely soluble in *methanol*, in *acetone* and in *glacial acetic acid*; soluble in *dichloromethane*; sparingly soluble in *chloroform* and in *water*.

Nicotinamide. Freely soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform* and in *ether.*

Nicotinic Acid. Soluble in boiling *water* and in boiling *ethanol (95 per cent)*; sparingly soluble in *water*; very slightly soluble in *chloroform*; practically insoluble in *ether.* It dissolves in dilute solutions of alkali hydroxides and carbonates.

Nicoumalone. Slightly soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *water* and in *ether.* It dissolves in aqueous solutions of alkali hydroxides.

Nifedipine. Freely soluble in *acetone* and in *chloroform*; sparingly soluble in *ethanol;* practically insoluble in *water*.

Nikethamide. Miscible with *water*, with *chloroform,* with *ethanol (95 per cent)* and with *ether*; slightly soluble in *carbon tetrachloride*.

Nimodipine. Freely soluble in *ethyl acetate*, sparingly soluble in *anhydrous ethanol*, practically insoluble in *water.*

Nitrazepam. Sparingly soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water.*

Nitrofurantoin. Soluble in *dimethylformamide*; very slightly soluble in *water* and in *ethanol (95 per cent).*

Nitrofurazone. Soluble in *dimethylformamide*; slightly soluble in *ethanol (95 per cent);* very slightly soluble in *water;* practically insoluble in *chloroform* and in *ether*.

Nitrous Oxide. At 20° and at a pressure of 101.3 kpa, 1 volume of gas dissolves in about 1.5 volumes of *water;* freely soluble in *ethanol (95 per cent);* soluble in *ether* and in oils.

Nitroxynil. Slightly soluble in *ethanol (95 per cent)*; sparingly soluble in *ether*; practically insoluble in *water.* Freely soluble in solutions of alkali hydroxide.

Noradrenaline Bitartrate. Freely soluble in *water;* slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Norethisterone. Soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water.*

Norfloxacin. Freely soluble in *acetic* acid; sparingly soluble in *chloroform*; slightly soluble in *acetone* and in *ethanol (95 per cent);* very slightly soluble in *water*, in *methanol* and in *ethyl acetate*; insoluble in *ether*.

Norgestimate. Freely soluble in *methylene chloride*; soluble in *acetone*; insoluble in *water.*

Norgestrel. Freely soluble in *chloroform* and in *dichloromethane*; sparingly soluble in *ethanol (95 per cent)*; insoluble in *water.*

Nortriptyline Hydrochloride. Freely soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *water* and in *methanol*; practically insoluble in *ether*, in *benzene* and in most other organic solvents.

Noscapine. Freely soluble in *chloroform*; soluble in *acetone*; slightly soluble in *ethanol (95 per cent)* and in *ether;* practically insoluble in *water.* It is soluble in strong acids. On dilution with *water,* the base may get precipitated.

Novobiocin Sodium. Freely soluble in *water,* in *ethanol (95 per cent)* and in *methanol.*

Nutmeg Oil. The material shall not require more than seven volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 27° when tested.

Nystatin. Freely soluble in *dimethylformamide*; slightly soluble in *methanol*; very slightly soluble in *water*; insoluble in *chloroform*, in *ether* and in *ethanol (95 per cent).*

Octyldodecanol. Miscible with *ethanol (95 per cent),* practically insoluble in *water.*

Oestradiol Benzoate. Sparingly soluble in *acetone*; slightly soluble in *ethanol (95 per cent)* and in fixed oils; practically insoluble in *water.*

Ofloxacin. Soluble in *glacial acetic acid*; slightly soluble in *water*, *dichloromethane* and *methanol.*

Olanzapine. Freely soluble in *dichloromethane;* practically insoluble in *water.*

Oleic Acid. Miscible with *dichloromethane,* with *chloroform,* with *ethanol (95 per cent)*, with *ether* and with light petroleum (boiling range 40° to 60°); practically insoluble in *water.*

Olmesartan Medoxomil. Slightly soluble in *ethanol (95 per cent),* practically insoluble in *heptane* and *water*.

Olopatadine Hydrochloride. Soluble in *dichloromethane,* sparingly soluble in *methanol* and in *alcohol*, very slightly soluble in *water*.

Omeprazole. Freely soluble in *dichloromethane* and in *chloroform*; soluble in *ethanol (95 per cent)* and in *methanol*; very slightly soluble in *water.* It dissolves in dilute solutions of alkali hydroxides.

Ondansetron. Very soluble in acid solutions and sparingly soluble in *water*.

Ondansetron Hydrochloride. Sparingly soluble in *water* and in *ethanol*; soluble in *methanol;* slightly soluble in *dichloromethane*.

Ormeloxifene Hydrochloride. Soluble in *chloroform* and in *methanol;* sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Ornidazole. Soluble in *chloroform* and in *methanol*.

Orphenadrine Citrate. Sparingly soluble in *water*, slightly soluble in *ethanol (95 per cent)*.

Oseltamivir Phosphate. Soluble in *water*.

Oxacillin Sodium. Freely soluble in *water*, soluble in *methanol*, practically insoluble in *dichloromethane*.

Oxaliplatin. Slightly soluble in *water*, very slightly soluble in *methanol*, insoluble in *ethanol (95 per cent)*.

Oxazepam. Slightly soluble in *alcohol* and in *chloroform*, very slightly soluble in *ether*, practically insoluble in *water*.

Oxcarbazepine. Soluble in *acetic acid*; sparingly soluble in *chloroform*; practically insoluble in *water*.

Oxetacaine. Freely soluble in *methanol*; soluble in *ethyl acetate*; practically insoluble in *water*.

Oxfendazole. Slightly soluble in *methanol*, in *dichloromethane*, *acetone* and in *ether*; practically insoluble in *water*.

Oxprenolol Hydrochloride. Very soluble in *water*, freely soluble in *ethanol (95 per cent)*; soluble in *chloroform*; practically insoluble in *ether*.

Oxybutynin Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*, soluble in *acetone*, practically insoluble in *cyclohexane*.

Oxyclozanide. Freely soluble in *acetone*; soluble in *ethanol (95 per cent)*; slightly soluble in *dichloromethane*; very slightly soluble in *water*.

Oxygen. At 20° and at a pressure of 101 kPa, 1 volume of gas dissolves in about 32 volumes of *water*.

Oxygen 93 Per cent. Soluble in *water* at 20° and at a pressure of 101 kPa.

Oxymetazoline Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*.

Oxytetracycline. Sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*. Dissolves in dilute acid and alkaline solutions.

Oxytetracycline Hydrochloride. Freely soluble in *water*, the solution becoming clouding on standing due to liberation of *oxytertracycline* base; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Oxytocin. Soluble in *water*, in *1-butanol* and in *2-butanol* (for solid).

Ozagrel Hydrochloride. Freely soluble in *water*, in *methanol*; very slightly soluble in *ethanol*; practically insoluble in *acetone*.

Paclitaxel. Soluble in *ethanol (95 per cent)*; insoluble in *water*.

Palmitic Acid. Soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Paliperidone. Sparingly soluble in *dichloromethane* and *0.1 M hydrochloric acid*; slightly soluble in *dimethyl-formamide* and *tetrahydrofuran* ; practically insoluble in *water*, *0.1 M sodium hydroxide* and *hexane*.

Pamidronate Disodium. Soluble in *water* and *2 M sodium hydroxide*, sparingly soluble in *0.1 M hydrochloric acid* and in *0.1 M acetic acid*, practically insoluble in organic solvents.

Pancretin. Soluble in *water* producing a slightly turbid solution; practically insoluble in *ethanol (95 per cent)*; and in *ether*.

D-Panthenol. Miscible with *water* and with *ethanol (95 per cent)*; soluble in *chloroform*; slightly soluble in *ether*; insoluble in fats and oils.

Pantoprazole. Freely soluble in *water* in *methanol* and in *ethanol*; practically insoluble in *hexane* and in *dichloromethane*.

Papain. Sparingly soluble in *water*; practically insoluble in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Paracetamol. Freely soluble in *ethanol*; sparingly soluble in *water*; very slightly soluble in *dichloromethane*.

Hard Paraffin. Soluble in *chloroform* and in *ether*; practically insoluble in *ethanol (95 per cent)* and in *water*.

Liquid Paraffin. Miscible with fixed and volatile oils. Soluble in *chloroform*, in *ether* and in *light petroleum* (40° to 60°); practically insoluble in *water* and in *ethanol (95 per cent)*.

Light Liquid Paraffin. Miscible with fixed and volatile oils. Soluble in *chloroform* and in *ether*; practically in soluble in *water* and in *ethanol (95 per cent)*.

White Soft Paraffin. Soluble in *chloroform*, in *ether* and in *light petroleum* (40° to 60°), the solutions sometimes show a slight opalescence; practically insoluble in *ethanol (95 per cent)* and in *water*.

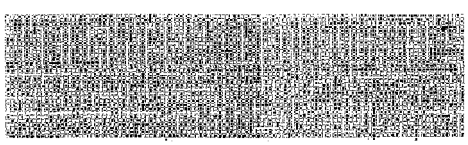
Yellow Soft Paraffin. Soluble in *chloroform*, in *ether* and in *light petroleum* (40° to 60°), the solutions sometimes show a slight opalescence; practically insoluble in *ethanol (95 per cent)* and in *water*.

Paraldehyde. Soluble in *water*; miscible with *ethanol (95 per cent)*, with *chloroform*, with *ether* and with volatile oils.

Parecoxib Sodium. Soluble in *water* and *dimethylformamide*; slightly soluble in *methanol*.

Paroxetine Hydrochloride. Freely soluble in *methanol*; sparingly soluble in *ethanol*; in *dichloromethane* and slightly soluble in *water*.

Paroxetine Hydrochloride Hemihydrate. Freely soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)* and in *methylene chloride*; slightly soluble in *water*.



Pemetrexed Disodium Heptahydrate. Freely soluble in *water*; very slightly soluble in *anhydrous ethanol*; practically insoluble in *methylene chloride*.

Penicillamine. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Pentamidine Isethionate. Soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Pentazocine. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; sparingly soluble in *ether*; practically insoluble in *water*.

Pentazocine Hydrochloride. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; practically insoluble in *ether*.

Pentazocine Lactate. Freely soluble in *methanol*; sparingly soluble in *chloroform*, in *ethanol (95 per cent)* and in *water*. It dissolves in aqueous solutions of alkali hydroxides.

Pentobarbitone Sodium. Very soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Pentoxifylline. Freely soluble in *chloroform* and in *methanol*, soluble in *water*; sparingly soluble in *ethanol* and slightly soluble in *ether*.

Peppermint Oil. The material shall not require more than seven volumes of *ethanol (70 per cent by volume)* to obtain a clear solution at 27° when tested.

Pepsin. Soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*. The solution in *water* may be slightly opalescent with a weakly acidic reaction.

Perindopril Erbumine. Freely soluble in *water* and in *ethanol (95 per cent)*, soluble or sparingly soluble in *methylene chloride*.

Perphenazine. Freely soluble in *dichloromethane*; soluble in *ethanol (95 per cent)*.

Pethidine Hydrochloride. Very soluble in *water*; freely soluble in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Petrolatum. Freely soluble in *benzene*, *carbon disulphide*, *chloroform*, and in turpentine oil; soluble in *ether*, solvent *hexane*, and in most fixed and volatile oils; practically insoluble in cold and hot *ethanol* and in cold *ethanol*; insoluble in *water*.

Phenindione. Freely soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)* and in *ether*; very slightly soluble in *water*. Solutions are yellow to red.

Pheniramine Maleate. Freely soluble in *water*, in *chloroform* and in *ethanol (95 per cent)*; very slightly soluble in *ether*.

Phenobarbitone. Soluble in *ethanol (95 per cent)* and in *ether*; sparingly soluble in *chloroform*; very slightly soluble in *water*. It is soluble in aqueous solutions of alkali hydroxides and carbonates.

Phenobarbitone Sodium. Freely soluble in *carbon dioxide free water* (a small fraction may be insoluble); soluble in *ethanol (95 per cent)*; practically insoluble in *dichloromethane* and in *ether*.

Phenol. Very soluble in *dichloromethane*, in *ethanol (95 per cent)* and in *glycerin*; soluble in *water*.

Phenolphthalein. Soluble in *ethanol (95 per cent)* and in *ether*; practically insoluble in *water*.

Phenoxyethanol. Miscible with *acetone*, with *ethanol (95 per cent)* and with *glycerol*; slightly soluble in *water*; in *arachis oil* and in *olive oil*.

Phenoxymethylpenicillin Potassium. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform*, in *ether*, in fixed oil and in *liquid paraffin*.

Phentolamine Mesylate. Freely soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform*.

Phenylalanine. Sparingly soluble in *water*; very slightly soluble in *methanol*, in *ethanol* and in dilute mineral acids.

Phenylephrine Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform*.

Phenylethyl Alcohol. Very soluble in *chloroform*, *ethanol (95 per cent)*, *ether*, *fixed oils*, *glycerine*, *propylene glycol*; slightly soluble in *mineral oil*, and *water*.

Phenylmercuric Acetate. Soluble in *acetone*; slightly soluble in *water* and in *ethanol (95 per cent)*.

Phenylmercuric Nitrate. Slightly soluble in *ethanol (95 per cent)* and in *glycerin*; very slightly soluble in *water*; practically insoluble in other usual organic solvents. It is more soluble in the presence of either *nitric acid* or alkali hydroxides.

Phenyramidol Hydrochloride. Freely soluble in *ethanol*; *methanol* and *water*.

Phenytoin. Sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *dichloromethane*; practically insoluble in *water*.

Phenytoin Sodium. Soluble in *water*, the solution showing some turbidity due to partial hydrolysis; soluble in *ethanol (95 per cent)*; practically insoluble in *dichloromethane* and in *ether*.

Pholcodine. Very soluble in *chloroform* and in *acetone*; freely soluble in *ethanol (95 per cent)*; slightly soluble in *ether*; sparingly soluble in *water*. It dissolves in dilute mineral acids.

Phosphoric Acid. Miscible with *water* and with *ethanol (95 per cent)*.

Physostigmine Salicylate. Soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *water*; very slightly soluble in *ether*.

Phytomenadione. Miscible with fatty oils, sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Pilocarpine Nitrate. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Pimozide. Soluble in *dichloromethane*; sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Pindolol. Slightly soluble in *methanol*; very slightly soluble in *chloroform*; practically insoluble in *water*. It dissolves in dilute mineral acids.

Pioglitazone Hydrochloride. Soluble in *dimethylformamide*; very slightly soluble in *acetronitrile*; practically insoluble in *water* and in *ether*.

Piperacillin. Freely soluble in *methanol*; slightly soluble in *water* and in *ethyl acetate*.

Piperacillin Sodium. Freely soluble in *water* and in *methanol*, practically insoluble in *ethyl acetate*.

Piperazine Adipate. Soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Piperazine Citrate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Piperazine Hydrate. Freely soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *ether*.

Piperazine Phosphate. Sparingly soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Piracetam. Freely soluble in *water*; soluble in *ethanol (95 per cent)*.

Pirfenidone. Soluble in *methanol*, *dimethylformamide* and *dimethylsulphoxide*.

Piroxicam. Slightly soluble in *ethanol (95 per cent)* and in aqueous alkaline solutions; very slightly soluble in *water*, in dilute acids and in most organic solvents.

Pitavastatin Calcium. Soluble in *dimethylsulphoxide*.

Plaster of Paris. Slightly soluble in *water*, the solubility decreasing sharply with rise of temperature; practically insoluble in *ethanol (95 per cent)*. It is more soluble in dilute mineral acids.

Polacrilin Potassium. Insoluble in *water* and in most liquids.

Poloxamers. Freely soluble in *water* and in *ethanol (95 per cent)*.

Polyethylene Glycol 1500. Freely soluble in *water* and in *chloroform*; sparingly soluble in *ethanol*; practically insoluble in *ether*.

Polyethylene Glycol 4000. Freely soluble in *water*, in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Polyethylene Glycol 6000. Freely soluble in *water*, in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Polymyxin B Sulphate. Freely soluble in *water*; slightly soluble in *ethanol*.

Polyoxy 35 Castor Oil. Very soluble in *ethanol (95 per cent)* and in *ethyl acetate*; insoluble in mineral acids.

Polysorbate 20. Miscible with *water*, with *ethanol*, with *ethyl acetate* and with *methanol*; practically insoluble in fixed oils and in *liquid paraffin*.

Polysorbate 80. Miscible with *water*, with *ethanol*, with *ethyl acetate* and with *methanol*; practically insoluble in fixed oils and in *liquid paraffin*.

Polyvinyl Acetate Phthalate. Soluble in *methanol* and in *ethanol (95 per cent)*; insoluble in *water*, in *dichloromethane*, and in *chloroform*.

Polyvinyl Alcohol. Soluble in *water*; slightly soluble in *ethanol (95 per cent)*; insoluble in organic solvents.

Potassium Chloride. Freely soluble in *water*, practically insoluble in *ethanol* and in *ether*.

Potassium Citrate. Very soluble in *water*, soluble in *glycerin*; practically insoluble in *ethanol (95 per cent)*.

Potassium Clavulanate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *acetone*.

Potassium Iodide. Very soluble in *water*; freely soluble in *glycerin*; soluble in *ethanol (95 per cent)*.

Potassium Permanganate. Freely soluble in boiling *water*; soluble in cold *water*.

Potassium Sorbate. Very soluble in *water*, slightly soluble in *ethanol (95 per cent)*.

Povidone. Freely soluble in *water*, in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Povidone Iodine. Soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform*, in *acetone* and in *ether*.

Pralidoxime Chloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Prasugrel Hydrochloride. Soluble in *water*; slightly soluble in *acetonitrile* and practically insoluble in *ethyl acetate*.

Pravastatin Sodium. Freely soluble in *water* and in *methanol*; soluble in *anhydrous ethanol (95 per cent)*.

Praziquantel. Freely soluble in *ethanol (95 per cent)*, and in *dichloromethane*; very slightly soluble in *water*.

Prazosin Hydrochloride. Slightly soluble in *methanol*, in *ethanol (95 per cent)*, *dimethylformamide* and in *dimethylacetamide*; very slightly soluble in *water*; practically in soluble in *chloroform* and in *acetone*.

Prednisolone. Soluble in *ethanol (95 per cent)* and in *methanol*; sparingly soluble in *acetone*; slightly soluble in *chloroform*; very slightly soluble in *water*.

Prednisolone Acetate. Slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*; practically insoluble in *water*.

Prednisolone Sodium Phosphate. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Prednisone. Slightly soluble in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Pregabalin. Sparingly soluble in *water*.

Pregelatinised Starch. It swells in cold *water*.

Prilocaine. Very soluble in *ethanol*; slightly soluble in *water*.

Primaquine Phosphate. Soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Probenecid. Soluble in *acetone*; sparingly soluble in *chloroform* and in *ethanol (95 per cent)*; slightly soluble in *ether*; practically insoluble in *water* and in dilute acids. It is soluble in dilute alkalis.

Procainamide Hydrochloride. Very soluble in *water*, freely soluble in *ethanol (95 per cent)*; slightly soluble in *acetone* and in *chloroform*; practically insoluble in *ether*.

Procaine Hydrochloride. Very soluble in *water*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; practically insoluble in *ether*.

Procaine Penicillin. Slightly soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Procarbazine Hydrochloride. Soluble in *water* and *methanol*; sparingly *soluble* in *ethanol*.

Prochlorperazine Maleate. Very slightly soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Prochlorperazine Mesylate. Very soluble in *water*, sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; practically insoluble in *ether*.

Procyclidine Hydrochloride. Soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; practically insoluble in *acetone* and in *ether*.

Progesterone. Freely soluble in *ethanol*; sparingly soluble in *acetone* and in fatty oils; practically insoluble in *water*.

Proguanil Hydrochloride. Soluble in *ethanol (95 per cent)*; slightly soluble in *water*; practically insoluble in *chloroform* and in *ether*.

Promazine Hydrochloride. Freely soluble in *water*, in *ethanol (95 per cent)* and in *dichloromethane*.

Promethazine Hydrochloride. Very soluble in *water*; freely soluble in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Promethazine Theoclate. Freely soluble in *chloroform*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*; practically insoluble in *ether*.

Propionic Acid. Soluble in organic solvents and in *ethanol (95 per cent)*.

Propofol. Miscible with *hexane* and with *methanol*. Very slightly soluble in *water*.

Propranolol Hydrochloride. Soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform*; practically insoluble in *ether*.

Propyl Gallate. Freely soluble in *ethanol (95 per cent)* and in *ether*; very slightly soluble in *water* and in arachis oil.

Propylene Glycol. Miscible with *water*, *acetone* and *chloroform*; soluble in *ether* and is immiscible with fixed oils.

Propylene Glycol Monocaprylate. Very soluble in *ethanol (95 per cent)*, *chloroform* and *methylene chloride*; practically insoluble in *water*.

Propyliodone. Soluble in *acetone*, *ethanol (95 per cent)*, and *ether*; practically insoluble in *water*.

Propylparaben. Freely soluble in *ethanol (95 per cent)*, in *acetone*, in *ether*, and in *methanol*; very slightly soluble in *water*.

Propylthiouracil. Sparingly soluble in *methanol*, in *ethanol (95 per cent)*; very slightly soluble in *ether* and in *water*. It dissolves in aqueous solutions of alkali hydroxides.

Propyphenazone. Freely soluble in *dichloromethane* and in *ethanol (95 per cent)*; soluble in *ether*; slightly soluble in *water*.

Protamine Sulphate. Sparingly soluble in *water*; practically insoluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*.

Prothionamide. Soluble in *ethanol (95 per cent)*, *methanol*; slightly soluble in *ether*, *chloroform*; practically insoluble in *water*.

Protriptyline Hydrochloride. Freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Pseudoephedrine Hydrochloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; sparingly soluble in *chloroform*.

Psoralen. Very soluble in *chloroform*; soluble in *ethanol (95 per cent)*; sparingly soluble in *ether*; practically insoluble in *light petroleum (60° to 80°)*.

Pyrantel Pamoate. Soluble in *dimethylsulphoxide*; practically insoluble in *water* and in *methanol*.

Pyrazinamide. Sparingly soluble in *water* and in *chloroform*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *ether*.

Pyridostigmine Bromide. Freely soluble in *water*, in *ethanol*, and in *chloroform*; slightly soluble in *hexane*; practically insoluble in *ether*.

Pyridoxine Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Pyrimethamine. Slightly soluble in *chloroform* and in *ethanol (95 per cent)*; very slightly soluble in *ether*; practically insoluble in *water*.

Quetiapine Fumarate. Slightely soluble in *water*, in *ethanol* and in *methanol*.

Quinalbarbitone Sodium. Freely soluble in *carbon dioxide free water* and in *ethanol (95 per cent)*; practically insoluble in *chloroform* and and in *ether*.

Quinapril Hydrochloride. Freely soluble in aqueous solvents.

Quinidine Sulphate. Soluble in *ethanol (95 per cent)* and in *chloroform*; sparingly soluble in *water*; practically insoluble in *ether*.

Quinine Bisulphate. Freely soluble in boiling *water* and in boiling *ethanol (95 per cent)*; soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*.

Quinine Dihydrochloride. Very soluble in *water*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; very slightly soluble in *ether*.

Quinine Sulphate. Freely soluble in a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol*; sparingly soluble in boiling *water* and in *ethanol (95 per cent)*; slightly soluble in *water*; very slightly soluble in *chloroform*; practically insoluble in *ether*.

Quiniodochlor. Freely soluble in *dimethylformamide* and in *pyridine*; practically insoluble in *ethanol (95 per cent)* and in *water*.

Rabeprazole Sodium. Soluble in *water*.

Racecadotril. Freely soluble in *methanol* and in *dichloromethane*; practically insoluble in *water*.

Rafoxanide. Soluble in *acetone*; sparingly soluble in *dichloromethane* and in *ethyl acetate*; slightly soluble in *methanol*; practically insoluble in *water*.

Raloxifene Hydrochloride. Freely soluble in *dimethylsulphoxide*; sparingly soluble in *methanol*; slightly soluble in *ethanol*; very slightly soluble in *water* and in *isopropyl alcohol*; practically insoluble in *ether* and in *ethyl acetate*.

Raltegravir Potassium. Soluble in *water*; slightly soluble in *methanol*; very slightly soluble in *acetonitrile* and in *ethanol*; and practically insoluble in *isopropyl alcohol*.

Ramelteon. Soluble in *methanol* and insoluble in *water*.

Ramipril. Freely soluble in *methanol*; sparingly soluble in *water*.

Ranitidine Hydrochloride. Freely soluble in *water*; soluble in *methanol* and in *ethanol (95 per cent)*; sparingly soluble in *ethanol*; very slightly soluble in *chloroform* and in *dichloromethane*.

Purified Rayon. Very soluble in *dilute sulphuric acid*; insoluble in ordinary solvents.

Rebamipide. Freely soluble in *dimethylformamide*, sparingly soluble in *sodium hydroxide solution*, slightly soluble in *methanol*; practically insoluble in *water* and *hydrochloric acid*.

Remdesivir. Soluble in *methanol*; sparingly soluble in *dimethylsulphoxide* and insoluble in *water*.

Repaglinide. Freely soluble in *methanol* and in *dichloromethane*; practically insoluble in *water*.

Reserpine. Freely soluble in *chloroform*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether* and in *water*.

Ribavirin. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; slightly soluble or very slightly soluble in *dichloromethane*.

Riboflavin. Very slightly soluble in *water*; more soluble in *saline solution* than in *water*; practically insoluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*.

Riboflavin Sodium Phosphate. Soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Rifabutin. Soluble in *chloroform* and in *methanol*; sparingly soluble in *alcohol* and very slightly soluble in *water*.

Rifampicin. Soluble in *chloroform* and in *methanol*; slightly soluble in *acetone*, in *ethanol (95 per cent)*, in *ether* and in *water*.

Rilpivirine. Slightly soluble in *dimethylformamide*.

Risedronate Sodium. Soluble in *water* and in aqueous solutions; insoluble in common organic solvents.

Risperidone. Soluble in *methylene chloride*; sparingly soluble in *alcohol*; practically insoluble in *water*.

Ritodrine Hydrochloride. Freely soluble in *water*; soluble in *ethanol*; practically insoluble in *acetone* and in *ether*.

Ritonavir. Freely soluble in *methanol* and in *ethanol (95 per cent)*; soluble in *2-propanol*.

Rivastigmine Tartrate. Very soluble in *water*, in *methanol* and very slightly soluble in *ethyl acetate*.

Rizatriptan Benzoate. Soluble in *methanol* and in *water*; sparingly soluble in *dimethylsulphoxide* and in *dimethylformamide*; slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*; insoluble in *toluene*, *cyclohexane* and in *hexane*.

Rocuronium Bromide. Freely soluble in *water* and in *ethanol*; very soluble in *methylene chloride*.

Roflumilast. Insoluble in *acetone* and *tetrahydrofuran*.

Ronidazole. Slightly soluble in *water*, in *ethanol (95 per cent)* and *dichloromethane*; very slightly soluble in *ether*.

Ropinirole Hydrochloride. Soluble in *water*.

Ropivacaine Hydrochloride. Soluble in *water*.

Rosemarry Oil. The material shall not require more than seven volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 27° when tested.

Rosuvastatin Calcium. Freely soluble in *acetonitrile*; soluble in *acetone*.

Roxithromycin. Freely soluble in *acetone*, in *ethanol (95 per cent)* and *dichloromethane*; very slightly soluble in *water*.

Rufinamide. Slightly soluble in *methanol* and insoluble in *water*.

Rupatadine Fumarate. Sparingly soluble in *methanol*.

Saccharin. Sparingly soluble in boiling *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform*, in cold *water* and in *ether*. It dissolves in *dilute ammonia solution*, in solutions of alkali hydroxides and in carbonates.

Saccharin Sodium. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Safinamide Methane Sulphonate. Soluble in *water* and *methanol*.

Salbutamol. Soluble in *ethanol (95 per cent)*; sparingly soluble in *water*; slightly soluble in *ether*.

Salbutamol Sulphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *ether*; very slightly soluble in *dichloromethane*.

Salicylic Acid. Freely soluble in *ethanol (95 per cent)* and in *ether*, sparingly soluble in *chloroform*; slightly soluble in *water*.

Salmeterol Xinafoate. Soluble in *methanol*; practically insoluble in *water*.

Saquinavir. Soluble in *methanol*.

Saquinavir Mesylate. Soluble in *methanol*.

Selegiline Hydrochloride. Freely soluble in *water* and in *methanol*, slightly soluble in *acetone*.

Secnidazole. Very soluble in *methanol*, in *ethanol*, in *chloroform*, in *acetic acid*; slightly soluble in *water*.

Seratrodast. Freely soluble in *dimethylsulphoxide*.

Sertaconazole Nitrate. Soluble in *methanol*; sparingly soluble in *ethanol (95 per cent)* and in *methylene chloride*; practically insoluble in *water*.

Sertraline Hydrochloride. Slightly soluble in *water*, *acetone* and in *2-propanol* and sparingly soluble in *ethanol*.

Shellac. Soluble in warm *ethanol (95 per cent)*; practically insoluble in *water*. Almost completely soluble in solutions of alkali hydroxides and of *borax*.

Sildenafil Citrate. Soluble in *dimethylformamide*, sparingly soluble in *acetic acid*, slightly soluble in *methanol*.

Colloidal Silicon Dioxide. Practically insoluble in *water* and in mineral acids with the exception of *hydrofluoric acid*. Dissolves in hot solutions of alkali hydroxides. When 1 g is shaken vigorously with 20 ml of *carbon tetrachloride* for 3 minutes; a transparent gel is produced.

Silver Nitrate. Very soluble in *water*; soluble in *ethanol (95 per cent)*.

Silver Sulphadiazine. Freely soluble in 30 per cent *ammonium solution*; slightly soluble in *acetone*; practically insoluble in *ethanol (95 per cent)*, *chloroform*, and *ether*.

Simvastatin. Very soluble in *dichloromethane*; freely soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Sisomicin Sulphate. Soluble in *dimethylsulphoxide*, in *methanol* and in *water*.

Sitagliptin Phosphate. Soluble in *water*; very slightly soluble in *ethanol*; practically insoluble in *heptane*.

Sodium Acetate. Very soluble in *water*; soluble in *ethanol (95 per cent)*.

Sodium Alendronate Trihydrate. soluble in *water*, practically insoluble in *methylene chloride* and in *methanol*.

Sodium Alginate. Slowly soluble in *water* forming a viscous, colloidal solution; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Sodium Aminosalicylate. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *chloroform* and in *ether*.

Sodium Ascorbate. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Sodium Benzoate. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Sodium Bicarbonate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Sodium Carbonate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Sodium Chloride. Freely soluble in *water* and slightly more soluble in boiling *water*; practically insoluble in *ethanol*.

Sodium Citrate. Freely soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Sodium Diatrizoate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone* and in *ether*.

Sodium Fluoride. Soluble in *water* practically insoluble in *ethanol (95 per cent)*.

Sodium Formaldehyde Sulphoxylate. Freely soluble in *water*; slightly soluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*.

Sodium Fusidate. Freely soluble in *water* and in *ethanol (95 per cent)*; slightly soluble in *chloroform*; practically insoluble in *acetone* and in *ether*.

Sodium Hydroxide. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*.

Sodium Lauryl Sulphate. Freely soluble in *water*, forming an opalescent solution; partly soluble in *ethanol (95 per cent)*.

Sodium Metabisulphite. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Sodium Methylparaben. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in fixed oils.

Sodium Nitrite. Freely soluble in *water*; sparingly soluble in *ethanol*.

Sodium Nitroprusside. Freely soluble in *water*, slightly soluble in *ethanol*.

Monobasic Sodium Phosphate. Very soluble in *water*; very slightly soluble in *ethanol*.

Sodium Phosphate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Sodium Propylparaben. Freely soluble in *water* and in *ethanol (50 per cent)*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in fixed oils.

Sodium Salicylate. Freely soluble in *water* (concentrated solutions are liable to deposit crystals of the hexahydrate) and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Sodium Starch Glycollate. Practically insoluble in *water*; insoluble in most organic solvents.

Sodium Stibogluconate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Sodium Thiosulphate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Sodium Valproate. Very soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Sofosbuvir. Very slightly soluble in *water*.

Solifenacin Succinate. Very soluble or freely soluble in *water*, soluble in *ethanol (95 per cent)* and practically insoluble in *heptane*.

Sorafenib Tosylate. Soluble in *dimethylsulphoxide*; insoluble in *water*.

Sorbic Acid. Freely soluble in *ethanol (95 per cent)* and in *ether*; slightly soluble in *water*.

Sorbitan Oleate. Soluble in fatty oils producing a hazy solution, miscible with *ethanol (95 per cent)*; practically insoluble but dispersible in *water*.

Sorbitol. Very soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Sorbitol Solution (70 Per cent) (Non- crystallising). Miscible with *water*, with *glycerin* and with *1,2-propanediol*.

Soyabean Oil. Miscible with *carbon disulphide*, *chloroform*, *ether* and *light petroleum*. Practically insoluble in *ethanol (95 per cent)* and *water*.

Spectinomycin Hydrochloride. Soluble in *water*; practically insoluble in *dichloromethane*, in *ethanol (95 per cent)* and in *ether*.

Spiramycin. Freely soluble in *ethanol*, in *methanol* and in *acetone*; sparingly soluble in *ether*; slightly soluble in *water*.

Spironolactone. Freely soluble in *chloroform*; soluble in *ethanol (95 per cent)*; slightly soluble in *ether*; practically insoluble in *water*.

Starch. Practically insoluble in cold *water* and in *ethanol (95 per cent)*.

Stavudine. Soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *dichloromethane*.

Stearic Acid. Soluble in *chloroform*, in *ethanol* and in *ether*; practically insoluble in *water*.

Stearyl Alcohol. Freely soluble in *chloroform* and in *ether*; soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Stilboestrol. Freely soluble in *ethanol (95 per cent)* and in *ether*; sparingly soluble in arachis oil; very slightly soluble in *water*. It is soluble in aqueous solutions of alkali hydroxides.

Streptokinase. Freely soluble in *water*.

Streptomycin Sulphate. Very soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Succinylcholine Chloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Sucralfate. Practically insoluble in *water* and in organic solvents. It dissolves in dilute mineral acids and in excess of caustic alkali solution.

Sucralose. Freely soluble in *water*, in *methanol*, and in *ethanol (95 per cent)*; slightly soluble in *ethyl acetate*.

Sucrose. Very soluble in *water*; freely soluble in *ethanol (70 per cent)*; practically insoluble in *ethanol*.

Sugar Spheres. Solubility in *water* varies according to the sugar-starch ratio.

Sulbactam Sodium. Freely soluble in *water*; sparingly soluble in *ethyl acetate*, very slightly soluble in *ethanol (95 per cent)*. It is freely soluble in dilute acids.

Sulfasalazine. Very slightly soluble in *ethanol (96 per cent)*, practically insoluble in *methylene chloride* and in *water*. It dissolves in dilute solutions of alkali hydroxides.

Sulphacetamide Sodium. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Sulphadiazine. Slightly soluble in *acetone*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *water*. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

Sulphadimidine. Soluble in *acetone*; slightly soluble in *ethanol*; very slightly soluble in *water*. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

Sulphadoxine. Slightly soluble in *ethanol (95 per cent)* and in *methanol*; very slightly soluble in *water*; practically insoluble in *ether*. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

Sulphamethizole. Soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*, in *chloroform* and in *ether*. It dissolves in dilute solutions of alkali hydroxides and in dilute mineral acids.

Sulphamethoxazole. Freely soluble in *acetone*; sparingly soluble in *ethanol (95 per cent)*, slightly soluble in *chloroform* and in *ether*; practically insoluble in *water*. It dissolves in dilute solutions of *sodium hydroxide*.

Sulphaquinoxaline. Freely soluble in aqueous solutions of alkalis; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water* and in *ether*.

Sulphathiazole Sodium. Freely soluble in *water*; soluble in *ethanol (95 per cent)*.

Sulpride. Sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)* and in *dichloromethane*; practically insoluble in *water*. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

Sumatriptan. Very slightly soluble in *water*.

Sumatriptan Succinate. Freely soluble in *water*; sparingly soluble in *methanol* and practically insoluble in *dichloromethane*.

Tacrolimus. Soluble in *methanol*, *ethanol and ethyl acetate*, sparingly soluble in *hexane* and insoluble in *water*.

Tadalafil. Freely soluble in *dimethyl sulphoxide*; slightly soluble in *dichloromethane*; practically insoluble in *water*.

Tafluprost. Slightly *soluble* in *dimethylformamide*, in *ethyl acetate*; insoluble in *water*.

Talc. Practically insoluble in *water* and in dilute solutions of acids and in alkali hydroxides.

Tamoxifen Citrate. Soluble in *methanol*; very slightly soluble in *water*, in acetone, in *chloroform* and in *ethanol (95 per cent)*.

Tamsulosin Hydrochloride. Freely soluble in *formic acid*; slightly soluble in *water* and in *anhydrous ethanol*.

Tapentadol Hydrochloride. Freely soluble in *methanol* and *water*.

Tartaric Acid. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*.

Tauroursodeoxycholic Acid. Soluble in *methanol*.

Tazobactam. Soluble in *dimethylformamide*; slightly soluble in *water*, in *methanol*, in *acetone*, and in *alcohol*; very slightly soluble in *ethyl acetate*, in *ethyl ether*, and in *chloroform*; insoluble in *hexane*.

Tazobactam Sodium. Freely soluble in *water*.

Teicoplanin. Freely soluble in *water*; sparingly soluble in *dimethylformamide*; practically insoluble in *ethanol (95 per cent)*.

Telmisartan. Sparingly soluble in *dichloromethane*; slightly soluble in *methanol*; practically insoluble in *water*.

Temozolomide. Soluble in *dimethylsulphoxide*; slightly soluble in *water* and practically insoluble in *acetonitrile*.

Teneligliptin Hydrobromide Hydrate. Freely soluble in *water*, soluble in *methanol*; insoluble in *cyclohexane*.

Tenofovir Alafenamide Fumarate. Soluble in *methanol*, very slightly soluble in *water* and practically insoluble in *ethyl acetate*.

Tenofovir Disoproxil Fumarate. Freely soluble in *dimethyl formamide* and *methanol*; sparingly soluble in *water*.

Tenoxicam. Sparingly soluble in *methylene chloride*; very slightly soluble in *ethanol (95 per cent)* and practically insoluble in *water*. It dissolves in solutions of acids and alkalis.

Terazosin Hydrochloride. Sparingly soluble in *water*; slightly soluble in *methanol*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*.

Terbinafine Hydrochloride. Freely soluble in *dehydrated alcohol* and in *methanol*; slightly soluble in *acetone*; very slightly or slightly soluble in *water*.

Terbutaline Sulphate. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Testosterone Propionate. Very soluble in *chloroform*; freely soluble in *acetone*, in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*, soluble in fixed oils.

Tetracycline. Soluble in *ethanol (95 per cent)* and in *methanol*; sparingly soluble in *acetone*; slightly soluble in *chloroform*; very slightly soluble in *water*; practically insoluble in *ether*. It dissolves in dilute acid and alkaline solutions.

Tetracycline Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform* and in *ether*. It dissolves in aqueous solutions of alkali hydroxides and carbonates.

Theophylline. Sparingly soluble in *ethanol*; slightly soluble in *chloroform* and *water*; very slightly soluble in *ether*. It dissolves in solutions of alkali hydroxides, in *aqueous ammonia* and in mineral acids.

Thiabendazole. Slightly soluble in *ethanol (95 per cent)*, in *dichloromethane*, in *chloroform* and in *ether*; practically insoluble in *water*. It dissolves in dilute mineral acid.

Thiacetazone. Soluble in *1,2-propanediol*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Thiamine Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Thiamine Mononitrate. Sparingly soluble in *water*; slightly soluble in *ethanol (95 per cent)* and in *methanol*.

Thiocolchicoside. Soluble in *water*; very slightly soluble in *ethanol (95 per cent)*.

Thioguanine. Freely soluble in dilute solutions of alkali hydroxides, insoluble in *water*, *alcohols* and *chloroform*.

Thiomersal. Freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Thiopentone Sodium. Freely soluble in *water*; partly soluble in *ethanol (95 per cent)*; practically insoluble in *benzene* and in *ether*.

Thiotepa. Freely soluble in *water*, in *ethanol (95 per cent)*, in *chloroform* and in *ether*.

Threonine. Freely soluble in *water*; insoluble in *ethanol*, in *ether* and in *chloroform*.

Thyme Oil. The material shall not require more than two volumes of *ethanol (70 percent by volume)* to obtain a clear solution at 20° when tested.

Thymol. Very soluble in *ethanol (95 per cent)* and in *ether*; freely soluble in volatile oils and in fixed oils; very slightly soluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Thyroxine Sodium. Slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*; practically insoluble in *ether*. It dissolves in aqueous solutions of alkali hydroxides.

Tibolone. Soluble in *acetone* and in *methanol*; practically insoluble in *water*.

Ticagrelor. Freely soluble in *methanol*; soluble in *ethanol*; practically insoluble in *water* and in *heptane*.

Ticarcillin Monosodium. Very soluble in *water*, soluble in *methanol* and very slightly soluble in *ethyl acetate*.

Tigecycline. Freely soluble in *water*; soluble in *1-octanol*; slightly soluble in *methanol*, in *ethanol*, and in *isopropyl alcohol*.

Timolol Maleate. Soluble in *water* and in *ethanol (95 per cent)*; sparingly soluble in *chloroform*; practically insoluble in *ether*.

Tinidazole. Soluble in *acetone* and in *dichloromethane*; sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)* and in *chloroform*; practically insoluble in *water*.

Tiotropium Bromide Monohydrate. Soluble in *methanol*; sparingly soluble in *water*; practically insoluble in *methylene chloride*.

Titanium Dioxide. Practically insoluble in *water* and in dilute mineral acids; slowly soluble in hot *sulphuric acid*.

Tizanidine Hydrochloride. Slightly soluble in *water* and *methanol*.

Tobramycin. Freely soluble in *water*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform* and in *ether*.

Tocopheryl Acetate. Freely soluble in *acetone*, in *chloroform* in *ethanol (95 per cent)*, in *ether* and in fixed oils; soluble in *ethanol*; practically insoluble in *water*.

Tofacitinib Citrate. Slightly soluble to practically insoluble in *methanol* and *water*; practically insoluble in *ethanol*.

Tolazamide. Freely soluble in *chloroform*; soluble in *acetone*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*.

Tolbutamide. Soluble in *ethanol (95 per cent)*, in *acetone* and in *chloroform*; slightly soluble in *ether*; practically insoluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Tolnaftate. Freely soluble in *acetone* and *in dichloromethane*; very slightly soluble in *ethanol (95 per cent)*; practically insoluble in *water*.

Tolterodine Tartrate. Sparingly soluble in *methanol*; slightly soluble in *ethanol (95 per cent)*.

Tolvaptan. Freely Soluble in *benzyl alcohol*; soluble in *methanol*; insoluble in *water*.

Tolu Balsam. Freely soluble in *ether*, in *ethanol (95 per cent)*, in *chloroform* and in solutions of fixed alkalies usually leaving some insoluble residue; insoluble in *water* and in *hexane*.

Topiramate. Freely soluble in *ethyl acetate* and in *ethanol*; slightly soluble in *water*.

Topotecan Hydrochloride. Soluble in *water*.

Torsemide. Slightly soluble in *0.1 M sodium hydroxide*, in *0.1 M hydrochloric acid*, in *ethanol (95 per cent)*, and in *methanol*; very slightly soluble in acetone and in *chloroform*; practically insoluble in *water* and in *ether*.

Tramadol Hydrochloride. Freely soluble in *water* and in *methanol*; very slightly soluble in *acetone*.

Trandolapril. Soluble in *methanol*, in *chloroform* and in *dichloromethane*.

Tranexamic Acid. Freely soluble in *water* and in *glacial acetic acid*, practically insoluble in *acetone* and in *ethanol (95 per cent)*.

Tranilast. Soluble in *ethanol*, in *dimethylformamide*; insoluble in *water*.

Travoprost. Very soluble in *methanol*, in *ethanol (95 per cent)*, in *acetonitrile* and in *chloroform*; insoluble in *water*.

Trazodone Hydrochloride. Sparingly soluble in *chloroform* and in *water*.

Triamcinolone. Sparingly soluble in *ethanol (95 per cent)*; slightly soluble in *water*; very slightly soluble in *chloroform* and in *ether*.

Triamcinolone Acetonide. Sparingly soluble in *chloroform* and in *ethanol (95 per cent)*; very slightly soluble in *ether*; practically insoluble in *water*.

Triamterene. Very slightly soluble in *water*, in *chloroform* and in *ethanol (95 per cent)*; practically insoluble in *water*.

Trichloromonofluoromethane. Soluble in *ethanol (95 per cent)*, in *ether* and in other organic solvents; practically insoluble in *water*.

Triclofos Sodium. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Triethyl Citrate. Miscible with *ethanol (95 per cent)*. Soluble in *water*; slightly soluble in fatty oils.

Tributyl Citrate. Freely soluble in *alcohol*, in *isopropyl alcohol*, in *acetone*, and in *toluene*, insoluble in *water*.

Trifluoperazine Hydrochloride. Freely soluble in *water*; soluble in *ethanol (95 per cent)*; slightly soluble in *chloroform*; insoluble in *ether* and in *benzene*.

Triflupromazine Hydrochloride. Soluble in *water*, in *ethanol (95 per cent)* and in *acetone*; insoluble in *ether*.

Trifluridine. Soluble in *water*.

Trimebutine Maleate. Soluble in *acetonitrile*, sparingly soluble in *acetone*; slightly soluble in *water and* in *ethanol (95 per cent)*.

Trimetazidine Hydrochloride. Freely soluble in *water*; sparingly soluble in *ethanol (95 per cent)*.

Trimethobenzamide Hydrochloride. Soluble in *water* and in warm *alcohol*, insoluble in *ether* and in *benzene*.

Trimethoprim. Sparingly soluble in *chloroform*; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water*; practically insoluble in *ether*.

Triprolidine Hydrochloride. Very soluble in *chloroform*; freely soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *ether*.

Tropicamide. Freely soluble in *chloroform*, in *ethanol (95 per cent)* and in solutions of strong acids; slightly soluble in *water*.

Trospium Chloride. Very soluble in *water*, freely soluble in *methanol* and practically insoluble in *dichloromethane*.

Troxidone. Very soluble in *chloroform*, in *ethanol (95 per cent)* and in *ether*; soluble in *water*.

Tryptophan. Soluble in hot *ethanol* and in *dilute hydrochloric acid*.

Tubocurarine Chloride. Soluble in *water* and in *ethanol (95 per cent)*; practically insoluble in *acetone*, in *chloroform* and in *ether*. It dissolves in solutions of alkali hydroxides.

Tylosin. Freely soluble in *methanol*; soluble in *ethanol (95 per cent)* and in *dichloromethane*; slightly soluble in *water*. It dissolve in dilute mineral acids.

Tylosin Tartrate. Freely soluble in *water* and in *dichloromethane*; slightly soluble in *ethanol (95 per cent)*;

practically insoluble in *ether*. It dissolves in dilute solutions of mineral acids.

Tyrothricin. Soluble in *ethanol (95 per cent)* and in *methanol*; practically insoluble in *water*.

Ulipristal Acetate. Freely soluble in *dichloromethane*, soluble in *methanol*; *acetone*, *ethanol* and insoluble in *water*.

Undecenoic Acid. Freely soluble in *chloroform*, in *ethanol (95 per cent)* in *ether* and in fixed and in volatile oils; practically insoluble in *water*.

Urea. Freely soluble in *water* and in boiling *ethanol (95 per cent)*; soluble in *ethanol (95 per cent)*; practically insoluble in *chloroform*, in *dichloromethane* and in *ether*.

Urokinase. Soluble in *water*.

Ursodeoxycholic Acid. Freely soluble in *ethanol (95 per cent)*; slightly soluble in *acetone*; practically insoluble in *dichloromethane* and in *water*.

Valacyclovir Hydrochloride. Soluble in *water*; insoluble in *dichloromethane*.

Valganciclovir Hydrochloride. Very slightly soluble in *ethanol*; practically insoluble in *2-propanol*, in *hexane*, in *acetone* and in *ethyl acetate*.

Valine. Soluble in *water*; practically insoluble in *ether*, in *ethanol* and in *acetone*.

Valproic Acid. Very soluble in organic solvents; slightly soluble in *water*.

Valsartan. Very soluble in *methanol*; practically insoluble in *water*.

Vancomycin Hydrochloride. Freely soluble in *water*; slightly soluble in *ethanol (95 per cent)*.

Vanillin. Freely soluble in *ethanol (95 per cent)* and in *methanol*; soluble in *ether*; slightly soluble in *water*. It dissolves in dilute solutions of alkali hydroxides.

Vasopressin. Soluble in *water*.

Vecuronium Bromide. Freely soluble in *dichloromethane*, sparingly soluble in *acetonitrile* and in *ethanol*; slightly soluble in *water*.

Venlafaxine Hydrochloride. Freely soluble in *water* and in *methanol*, soluble in anhydrous *ethanol*, slightly soluble or practically insoluble in *acetone*.

Verapamil Hydrochloride. Freely soluble in *chloroform*; soluble in *water*; sparingly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Vildagliptin. Freely soluble in *water*, soluble in *ethanol*, *dimethyl formamide* and in *dimethyl sulphoxide*.

Vinblastine Sulphate. Freely soluble in *water*; soluble in *methanol*; practically insoluble in *ethanol (95 per cent)* and in *ether*.

Vincristine Sulphate. Freely soluble in *water*; soluble in *methanol*; slightly soluble in *ethanol (95 per cent)*; practically insoluble in *ether*.

Vinorelbine Tartrate. Freely soluble in *water*.

Vinpocetine. Soluble in *dichloromethane*, slightly soluble in *ethanol* and practically insoluble in *water*.

Vitamin A Concentrate (Oily Form). Miscible with organic solvents. Soluble or partly soluble in *ethanol*; practically insoluble in *water*. Partial crystallisation may occur in highly concentrated solutions.

Vitamin A Concentrate (Powder Form). Depending on the formulation, may be practically insoluble in *water*, swell or form an emulsion.

Voglibose. Soluble in *water* and slightly soluble in *methanol*.

Voriconazole. Freely soluble in *acetone* and in *dichloromethane*; very slightly soluble in *water*.

Warfarin Sodium. Very soluble in *water* and in *ethanol (95 per cent)*; soluble in *acetone*; very slightly soluble in *dichloromethane* and in *ether*.

Warfarin Sodium Clathrate. Very soluble in *water*; freely soluble in *ethanol (95 per cent)*; very slightly soluble in *dichloromethane* and in *ether*.

Wool Fat. Soluble in *chloroform* and in *ether*; slightly soluble in boiling *ethanol (95 per cent)*; practically insoluble in *water*.

Hydrous Wool Fat. Soluble in *chloroform* and in *ether*, with the separation of *water*; practically insoluble in *water*.

Xanthan Gum. Soluble in *water* and practically insoluble in organic solvents.

Xylazine Hydrochloride. Very soluble in *methanol*; freely soluble in *water* and in *methylene chloride*.

Xylometazoline Hydrochloride. Freely soluble in *ethanol (95 per cent)*; soluble in *water* and in *chloroform*; practically insoluble in *ether*.

Xylose. Very soluble in *water*; soluble in hot *ethanol (95 per cent)*.

Zaleplon. Sparingly soluble in *alcohol*, slightly soluble in *propylene glycol* and practically insoluble in *water*.

Zanamivir. Sparingly soluble in *water*; insoluble in *acetone*, in *acetonitrile*, in *ethanol* and in *isopropyl alcohol*.

Zidovudine. Soluble in *ethanol (95 per cent)* and in *methanol*; sparingly soluble in *water*.

Zinc Chloride. Very soluble in *water*; freely soluble in *ethanol (95 per cent)* and in *glycerin*.

Zinc Citrate. Soluble in *dilute hydrochloric acid*; insoluble in *water*.

Zinc Oxide. Practically insoluble in *water* and in *ethanol (95 per cent)*. It dissolves in dilute mineral acids.

Zinc Stearate. Practically insoluble in *water*, in *ethanol (95 per cent)* and in *ether*.

Zinc Sulphate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Zinc Sulphate Monohydrate. Very soluble in *water*; practically insoluble in *ethanol (95 per cent)*.

Zinc Undecenoate. Practically insoluble in *water*, in *ethanol (95 per cent)* and in *ether*.

Ziprasidone Hydrochloride Monohydrate. Slightly soluble in *methanol* and in *dichloromethane*; practically insoluble in *water*.

Zoledronic Acid. Slightly soluble in *water*.

Zolmitriptan. Freely soluble in *methanol*, *dimethylsulphoxide* and in *dimethylformamide*; soluble in *ethanol (95 per cent)*, *acetone* and in *isopropyl alcohol*; sparingly soluble in *acetonitrile*, in *ethyl acetate* and in *dichloromethane*; slightly soluble in *chloroform*; insoluble in *toluene*, in *cyclohexane* and in *hexane*.

Zolpidem Tartrate. Sparingly soluble in *methanol*; slightly soluble in *water*; practically insoluble in *dichloromethane*.

Zonisamide. Free soluble in *dimethylformamide*; soluble in *methanol*.

Zopiclone. Freely soluble in *dichloromethane*; sparingly soluble in *acetone*; practically insoluble in *ethanol (95 per cent)* and in *water*. It dissolves in dilute mineral acids.

Zuclopenthixol Acetate. Very soluble in *dichloromethane*, in *ethanol (95 per cent)* and in *ether*; very slightly soluble in *water*.

Zuclopenthixol Decanoate. Very slightly soluble in *water*, very soluble in *ethanol (95 per cent)* and in *dichloromethane*.

2.4.27. Refractive Index

The refractive index (n) of a substance with reference to air is the ratio of the sin of the angle of incidence to the sin of the angle of refraction of a beam of light passing from air into the substance. It varies with the wavelength of the light used in its measurement.

Unless otherwise specified in the individual monograph, the refractive index, n_D^{20} is measured at 20° ± 0.5° with reference to the wavelength of the D-line of sodium (λ = 589.3 nm). The temperature should be carefully adjusted and maintained since the refractive index varies significantly with temperature.

The Abbe refractometer is convenient for most measurements of refractive index but other refractometers of equal or greater accuracy may be used. Commercial refractometers are normally constructed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium. The apparatus is provided with a water jacket to control the temperature of measurements. The manufacturer's instructions relating to a suitable light source should be followed subject to the directions given in the Pharmacopoeia.

To achieve the theoretical accuracy of ± 0.0001, it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25° or against the reference liquids given in the following table.

Table

Reference liquid	n_D^{20}	Temperature coefficient $\Delta n/\Delta t$
Carbon tetrachloride	1.4603	-0.00057
Toluene	1.4969	-0.00056
α-Methylnaphthalene	1.6176	-0.00048

*Refractive index value for the D line of sodium measured at 20°.

NOTE — The cleanliness of the instrument should be checked frequently by determining the refractive index of distilled water.

2.4.28. Viscosity

The determination of viscosity of newtonian liquids is carried out by means of a capillary viscometer, unless otherwise specified; Methods A and B described below are recommended. For non-newtonian liquids Method C using the rotating viscometer may be used.

For measurement of viscosity, the temperature of the substance being measured must be accurately controlled, since small temperature changes may lead to marked changes in viscosity. For usual pharmaceutical purposes, the temperature should be maintained to within ± 0.1°.

Method A (Using Ostwald-type Viscometer)

Apparatus

The apparatus consists of a glass U-tube viscometer (see Fig. 2.4.28-1) made of clear borosilicate glass and constructed in accordance with the dimensions shown in the figure and in Table 1.

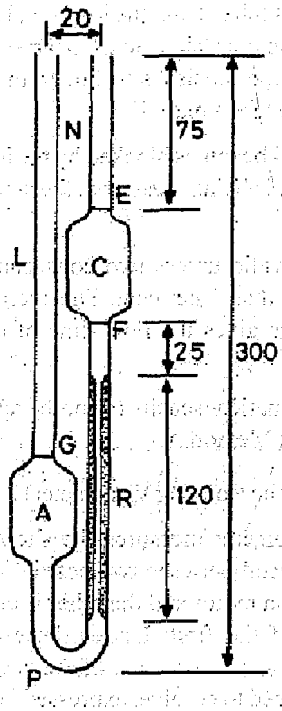
Procedure. Fill the viscometer, previously washed and completely dried, with the liquid under examination through tube L to slightly above the mark G, using a long pipette to minimise wetting the tube above the mark. Place the tube vertically in a water-bath maintained at the temperature indicated in the monograph and allow to stand for not less than 30 minutes to allow the temperature to reach equilibrium.

Table 1 – U-Tube Viscometer Dimensions

Size	National viscometer constant	Kinematic viscosity range	Inside diameter of tube R	Outside diameter of tubes*		Volume of bulb C	Vertical distance F to G	Outside diameter of bulbs A and C
				L and PN				
	m^2s^{-2}	mm^2s^{-1}	mm (± 2 per cent)	mm	mm	ml (± 5 per cent)	mm	mm
A**	0.003	0.9 to 3	0.50	8 to 9	6 to 7	5.0	91 ± 4	21 to 23
B	0.01	2.0 to 10	0.71	8 to 9	6 to 7	5.0	87 ± 4	21 to 23
C	0.03	6 to 30	0.88	8 to 9	6 to 7	5.0	83 ± 4	21 to 23
D	0.1	20 to 100	1.40	9 to 10	7 to 8	10.0	78 ± 4	25 to 27
E	0.3	60 to 300	2.00	9 to 10	7 to 8	10.0	73 ± 4	25 to 27
F	1.0	200 to 1000	2.50	9 to 10	7 to 8	10.0	70 ± 4	25 to 27
G	3.0	600 to 3000	4.00	10 to 11	9 to 10	20.0	60 ± 3	32 to 35
H	10.0	2000 to 10,000	6.10	10 to 11	9 to 10	20.0	50 ± 3	32 to35

* Use 1 to 1.25 mm wall tubing for N, P and L.

** 300 s minimum flow time; 200 s minimum flow time for all other sizes.



(Dimension in mm; tolerances ± 10 per cent).

Fig. 2.4.28-1: Ostwald-Type Viscometer

Adjust the volume of the liquid so that the bottom of the meniscus settles at the mark G. Suck or blow the liquid to a point about 5 mm above the mark E. After releasing pressure or suction, measure the time taken for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F.

Calculate, as required, either the kinematic viscosity (v) in square millimetres per second ($mm^2 s^{-1}$) from the expression

$$v = Kt,$$

or the dynamic viscosity (n) in millipascal seconds (mPa s) from the expression

$$n = KPt,$$

where, t = time in seconds for the meniscus to fall from E to F,

P = mass/volume (g cm^{-3}) obtained by multiplying the relative density (2.4.29), of the liquid under examination by 0.998203.

The constant (K) of the instrument is determined on a liquid of known viscosity.

NOTE — For Dextran Injections, the viscosity ratio is calculated by dividing the time taken with the liquid under examination by the time taken by saline solution for the meniscus to fall from E to F.

Method B (Using the Suspended-level Viscometer)

Apparatus

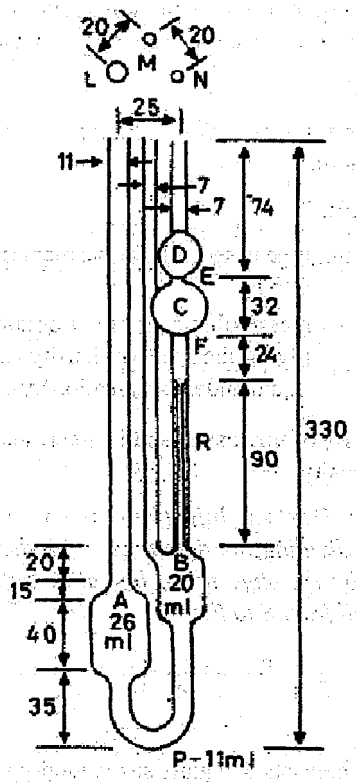
The apparatus consists of a glass suspended-level viscometer (see Fig. 2.4.28-2) made of clear borosilicate glass and constructed in accordance with the dimensions shown in the figure and in Table 2.

Procedure. Fill the viscometer through tube L with a sufficient quantity of the liquid under examination to ensure that bulb A is satisfactorily filled without blocking the ventilation tube M. After the tube has been placed vertically in a bath maintained at the specified temperature allow it to stand for not less than

Table 2 – Suspended-level Viscometer- Dimensions

Size	National viscometer constant m^2s^{-2}	Kinematic viscosity range mm^2s^{-1}	Inside diameter of tube R mm (2 per cent)	Volume of bulb C mm	Inside diameter of tube N mm
1*	0.01	3.5 to 10	0.64	5.6	2.8 to 3.2
1A	0.03	6 to 30	0.84	5.6	2.8 to 3.2
2	0.1	20 to 100	1.15	5.6	2.8 to 3.2
2A	0.3	60 to 300	1.51	5.6	2.8 to 3.2
3	1.0	200 to 1100	2.06	5.6	3.7 to 4.3
3A	3.0	600 to 3000	2.74	5.6	4.6 to 5.4
4	10.0	2000 to 10,000	3.70	5.6	4.6 to 5.4
4A	30.0	6000 to 30,000	4.97	5.6	5.6 to 6.4
5	100.0	20,000 to 100,000	6.76	5.6	6.8 to 7.5

* 350 s minimum flow times; 200 s minimum flow time for all other sizes.



(Dimensions in mm; tolerances ± 10 per cent or ± 10 mm, whichever is less)

Fig. 2.4.28-2: Suspended-Level Viscometer

30 minutes to allow the temperature to reach equilibrium, close tube M and apply suction to tube N until the liquid reaches a level about 5 mm above mark E. Hold the liquid at this level by closing tube N and open tube M. When the liquid is clear of the capillary end of tube N and the lower end of tube M, open tube N. Measure the time taken, to the nearest 0.2 of a second, for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F.

If the end of tube M becomes blocked by the liquid at any time while the flow time is being measured, the determination must be repeated.

The result is not valid unless two consecutive readings do not differ by more than 1 per cent. The average of not fewer than three readings gives the flow time of the liquid under examination.

Calculate the kinematic viscosity (v) or the dynamic viscosity (h) as given under Method A.

Method C (Using the Rotating Viscometer)

The rotating viscometer measures the shearing forces in a liquid medium placed between two coaxial cylinders one of which is driven by a motor and the other is caused to revolve by the rotation of the first. Under these conditions, the viscosity becomes a measurement of the angle of deflection of the cylinder caused to revolve, expressed in newton metres.

The principle of the method is to measure the force acting on a rotor (torque) when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotating viscometers are used for measuring the viscosity of Newtonian (shear-independent viscosity) or non-Newtonian liquids (shear dependent viscosity or apparent viscosity). Rotating viscometers can be divided in 2 groups, namely absolute and relative viscometers. In absolute viscometers the flow in the measuring geometry is well defined. The measurements result in absolute viscosity values, which can be compared with any other absolute values.

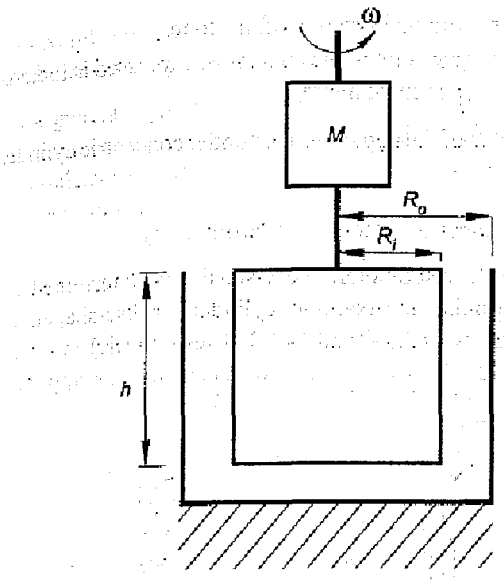


Fig. 2.4.28-3

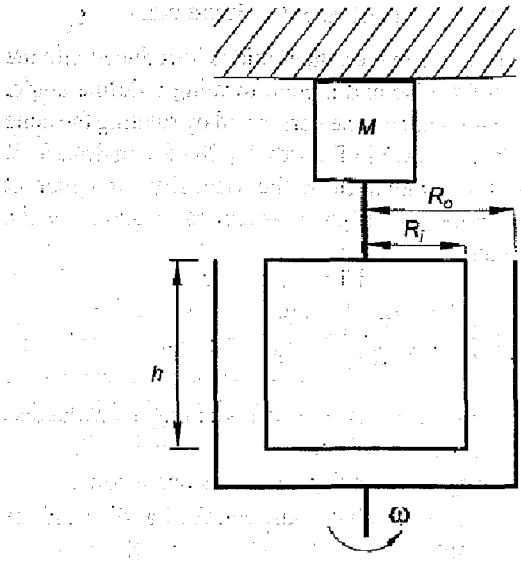


Fig. 2.4.28-4

In relative viscometers the flow in the measuring geometry is not defined. The measurements result in relative viscosity values, which cannot be compared with absolute values or other relative values if not determined by the same relative viscometer method.

Different measuring systems are available for given viscosity ranges as well as several rotational speeds.

Apparatus

The following types of instruments are most common.

Concentric Cylinder Viscometers (Absolute Viscometers)

In the concentric cylinder viscometer (coaxial double cylinder viscometer or simply coaxial cylinder viscometer), the viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Viscosity measurement can be performed by rotating the inner cylinder (Searle type viscometer) or the outer p (Couette type viscometer), as shown in Fig. 2.4.28.-3 and 2.4.28.-4, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds is given by the following formula:

$$\eta = \frac{1}{\omega}\left(\frac{M}{4\pi h}\right)\left(\frac{1}{R_i^2} - \frac{1}{R_o^2}\right) = k\frac{M}{\omega}$$

Where, M = torque in newton-metres acting on the cylinder surface,
ω = angular velocity in radians per second,
h = height of immersion in metres of the inner cylinder in the liquid medium,
R_i = radius in metres of the inner cylinder,
R_o = radius in metres of the outer cylinder,
k = constant of the apparatus, expressed in radians per cubic metre.

For non-Newtonian liquids it is indispensable to specify the shear stress (τ) or the shear rate (γ) at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute viscometers), there is a proportional relationship between M and τ and also between ω and γ:

$$\tau = AM \qquad \gamma = B\omega$$

where A and B are constants for the instrument and are calculated from the following expressions:

for concentric surface:

$$A = \frac{1}{4\pi h}\frac{R_i^2 + R_o^2}{R_i^2 R_o^2} \qquad B = \frac{R_i^2 + R_o^2}{R_o^2 - R_i^2}$$

for cone-plates:

$$A = \frac{3}{2\pi R^3} \qquad B = \frac{1}{\alpha}$$

where, M = torque in Newton-metres acting on the cone or cylinder surface,
ω = angular velocity in radians per second,
R_i = radius in metres of the inner cylinder,
R_0 = radius in metres of the outer cylinder,
R = radius in metres of the cone,
h = height of immersion in metres of the inner cylinder in the liquid medium,
τ = angle in radians between the flat disk and the cone,
γ = shear stress in pascals (Pa),

Cone-Plate Viscometers (Absolute Viscometers)

In the cone-plate viscometer, the liquid is introduced into the gap between a flat disc and a cone forming a define angle. Viscosity measurement can be performed by rotating the cone or the flat disc, as shown in Figures 2.4.28.-5 and 2.4.28.-6, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds is given by the following formula:

$$\eta = \left(\frac{M}{\omega}\right)\left(\frac{3\alpha}{2\pi R^3}\right) = k\frac{M}{\omega}$$

Where, M = torque in Newton-metres acting on the flat disc or cone surface,

ω = angular velocity in radians per second,

α = angle in radians between the flat disc and the cone,

R = radius in metres of the cone,

k = constant of the apparatus, expressed in radians per cubic metre.

Constants A, B of the apparatus (see under concentric cylinder viscometers).

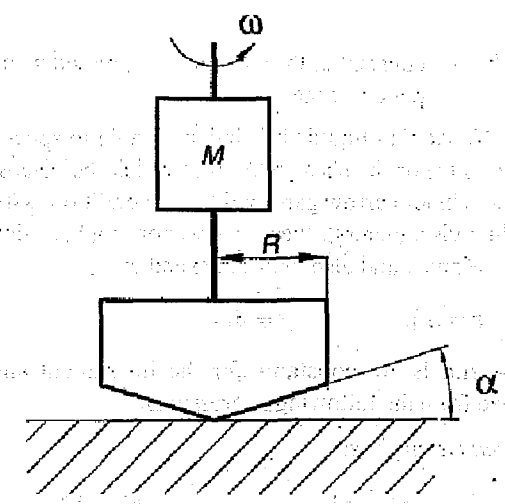


Fig. 2.4.28-5

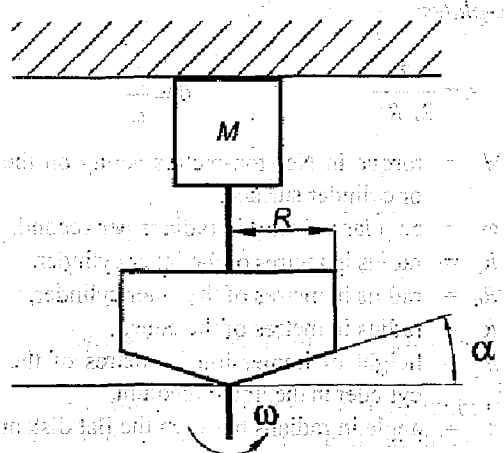


Fig. 2.4.28-6

Spindle Viscometers (Relative Viscometers)

In the spindle viscometer, the viscosity is determined by rotating a spindle (for example, cylinder- or disc-shaped, as shown in Figures 2.4.28.-7 and 2.4.28.-8, respectively) immersed in the liquid. Relative values of viscosity (or apparent

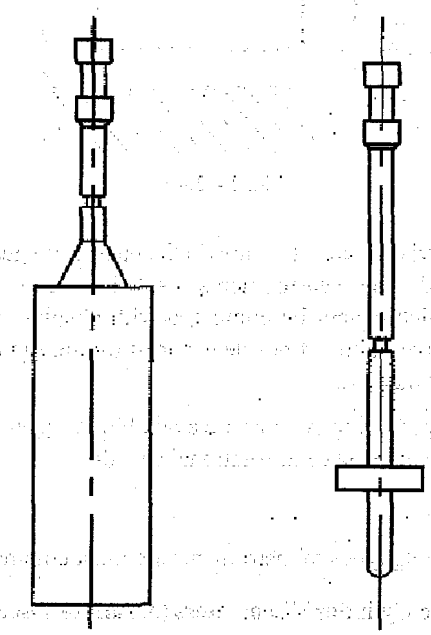


Fig. 2.4.28-7

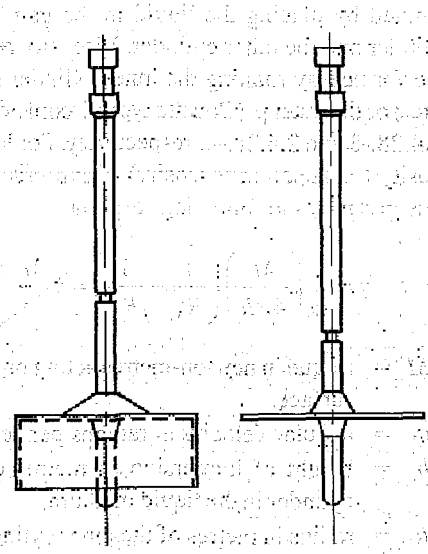


Fig. 2.4.28-8

viscosity) can be directly calculated using conversion factors from the scale reading at a given rotational speed.

In a general way, the constant k of the apparatus may be determined at various speeds of rotation using a certified viscometer calibration liquid. The viscosity ç then corresponds to the formula.

$$\eta = k\frac{M}{\omega}$$

Procedure

Measure the viscosity (or apparent viscosity) according to the instructions for the operation of the rotating viscometer. Since small temperature changes may lead to marked changes in viscosity, the temperature should be held to within ± 0.1°. The temperature for measuring the viscosity is indicated in the monograph. For non-Newtonian systems, the monograph indicates the type of viscometer to be used and if absolute viscometers are used the angular velocity or the shear rate at which the measurement is made. If it is impossible to obtain the indicated shear rate exactly, use a shear rate slightly higher and a shear rate slightly lower and interpolate.

With relative viscometers the shear rate is not the same throughout the sample and therefore it cannot be defined. Under these conditions, the viscosity of non-Newtonian liquids determined from the previous formula has a relative character, which depends on the type of spindle and the angular velocity as well as the dimensions of the sample container (Ø = minimum 80 mm) and the depth of immersion of the spindle. The values obtained are comparable only if the method is carried out under experimental conditions that are rigorously the same.

2.4.29. Weight Per Millilitre and Relative Density (Specific Gravity)

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. When the substance is a solid at 25°, determine the specific gravity of the melted material at the temperature directed in the individual monograph, and refer to water at 25°.

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter (1 kg/m^3 = 10^{-3} g/cm^3).

Unless otherwise directed in the individual monograph, use Method A.

Method A

Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled water contained in it at 25°. Adjust the temperature of the liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, filled pycnometers must be brought to the temperature of the balance before they are weighed. Subtract the tare weight of the pycnometer from the filled weight.

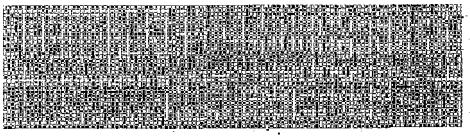
The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25°, unless otherwise directed in the individual monograph.

Alcohol Table

Relative density At 25°	Per cent ethanol w/w at 15.56°	Per cent ethanol v/v at 15.56°
0.8158	90.0	93.3
0.8146	90.5	93.6
0.8131	91.0	94.0
0.8118	91.5	94.3
0.8104	92.0	94.7
0.8090	92.5	95.0
0.8076	93.0	95.4
0.8062	93.5	95.8
0.8048	94.0	96.1
0.8034	94.5	96.5
0.8020	95.0	96.8
0.8006	95.5	97.1
0.7992	96.0	97.5
0.7977	96.5	97.8
0.7962	97.0	98.1
0.7977	97.5	98.4
0.7932	98.0	98.8
0.7917	98.5	99.1
0.7902	99.0	99.4
0.7886	99.5	99.7
0.7871	100.0	100.0

Method B

The procedure includes the use of the *Oscillating transducer density meter.* The apparatus consists of the following:



- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;
- a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants A and B described below; and
- a means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested.

The oscillation period is a function of the spring constant (c) and the mass of the system:

$$T^2 = \{(M/c) + (\rho \times V)/c\} \times 4\pi^2$$

where ρ is the density of the liquid to be tested, M is the mass of the tube, and V is the volume of the filled tube.

Introduction of two constants $A = c/(4\pi^2 \times V)$ and $B = (M/V)$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\frac{\rho_{(L)}}{\rho_{(W)}}$$

where $\rho_{(L)}$ and $\rho_{(W)}$ are the densities of the liquid and water, respectively, both determined at 25, unless otherwise directed in the individual monograph.

Calibration. The constants A and B are determined by operating the instrument with the U-tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water: the results displayed for the control measurement using degassed water do not deviate from the reference value (ρ_{25} = 0.997043 g per cm^3) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the order of 1×10^{-3} g per cm^3 to 1×10^{-5} g per cm^3 and a repeatability of 1×10^{-4} g per cm^3 to 1×10^{-6} g per cm^3. For example, an instrument specified to $\pm 1 \times 10^{-4}$ g per m^3 must display 0.9970 ± 0.0001 g per cm^3 in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

Procedure. Using the manufacturer's instructions, perform the measurements using the same procedure as for *Calibration*. If necessary, equilibrate the liquid to be examined at 25 before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- temperature uniformity throughout the tube,
- nonlinearity over a range of density,
- parasitic resonant effects, and
- viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence.

2.4.30. Total Organic Carbon in Water

This method for determining total organic carbon (TOC) indirectly measures the total amount of organic substances present in water for pharmaceutical use. The molecules of organic matter in water are oxidised to produce carbon dioxide which is then measured in an instrument and from the result, the concentration of carbon in the water is calculated. The determination of carbon in water may be made either on-line (in the line of supply of the water) or off-line.

Irrespective of the method used, the system is qualified by analysing a standard solution of a substance that is easily oxidisable (such as sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured, and by interpreting the results in limit tests. The suitability of the system is determined by analysis of a solution prepared with a substance that is oxidisable with difficulty (such as *1,4-benzoquinone*).

Apparatus. Any suitable apparatus capable of discriminating between organic and inorganic carbon either by purging inorganic carbon from the sample under examination before oxidisation or by the measurement of the inorganic carbon and subtraction from the total carbon, may be used.

The instrument manufacturer's instructions should be followed for installation and subsequent operations. The instrument should be calibrated and the system suitability should be verified at suitable intervals. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

Glassware. Use glassware that has been thoroughly cleaned by a method that will remove organic matter (5.1). Use TOC *water* for the final rinse of glassware.

Solutions

TOC water. Highly purified *water* complying with the following specifications:

Conductivity. Not more than 1.0 µS cm^{-1} at 25°

TOC. Not more than 0.1 mg/l

Test solution. Collect carefully the water to be tested in an airtight container with minimum head space and examine it with minimum delay.

Standard solution. Dissolve *sucrose*, previously dried at 105° for 3 hours, in sufficient TOC water to produce a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

System suitability solution. Dissolve *1,4-benzoquinone* in sufficient *TOC water* to produce a solution containing 0.75 mg of *1,4-benzoquinone* per litre (0.50 mg of carbon per litre).

NOTE — Use TOC water obtained at the same time as that used to prepare the standard solution and the system suitability solution.

Control solutions. Prepare suitable blank solutions or other solutions needed for establishing the base for calibration adjustments. Run the appropriate blanks for zeroing the instrument.

System suitability. Run successively the TOC water, standard solution and system suitability solution and record the responses r_w, r_s and r_{ss}, respectively. Calculate the percentage response efficiency from the expression:

$$\frac{r_{ss} - r_w}{r_s - r_w} \times 100$$

The system is suitable if the response efficiency is not less than 85 per cent and not more than 115 per cent of the theoretical response.

Procedure. Run the test solution and record the response, r_t. The test solution complies with the test if r_t is not greater than r_s-r_w.

2.4.31. Thermal Analysis

Introduction

Thermal analysis is the technique to measure the physical–chemical properties of materials as a function of temperature. Thermal analysis is a group of techniques in which the variation of a physical property of a substance is measured as a function of temperature. The most commonly used techniques are those which measure changes of mass or changes in energy of a sample of a substance. Furthermore, they may provide information on desolvation, dehydration, decomposition, crystal perfection, polymorphism, melting temperature, sublimation, glass transitions, evaporation, pyrolysis, solid-solid interactions, and purity. Such data are useful in the characterization of substances with respect to compatibility, stability, packaging, and quality control. The measurements used most often in thermal analysis, i.e., transition and melting point temperatures by differential scanning calorimetry (DSC), mass change of a substance by thermo-gravimetric analysis, hot-stage microscopy and eutectic impurity analysis are described here.

Thermo Gravimetric Analysis (TGA)

Thermo-gravimetric techniques involves the determination of the mass of a substance as a function of temperature, or time of heating, or both according to a controlled temperature programme. It is often used to investigate dehydration/desolvation processes and compound decomposition. (TGA) provides more useful information than loss on drying at fixed temperature, often for a fixed time and in what is usually an ill-defined atmosphere. Usually, loss of surface-absorbed solvent can be distinguished from solvent in the crystal lattice and from degradation losses. The measurements can be carried out in atmospheres having controlled humidity and oxygen concentration to reveal interactions with the drug substance, between drug substances, and between active substances and excipients or packaging materials.

Apparatus. TG apparatus consists of a thermo-balance are a device for heating or cooling the substance according to a given temperature program, a sample holder in a controlled atmosphere, an electro-balance and a recorder. In some cases the instrument may be coupled to a device permitting the analysis of volatile products.

Calibration. Calibration is required with all systems, like the mass scale is calibrated by the use of standard weights.

Temperature verification. Check the temperature scale using a suitable material according to the manufacturer's instructions. Temperature calibration is performed by analyzing a high-purity magnetic standard such as nickel for its curie temperature and comparing the measured value to the theoretical value.

Verification of the electro-balance. Place a suitable quantity of a suitable certified reference material (for example, calcium oxalate monohydrate RS) in the sample holder and record the mass. Set the heating rate according to the manufacturer's instructions and start the temperature increase. Record the thermo-gravimetric curve as a graph with temperature, or time, on the abscissa, increasing from left to right, and mass on the ordinate, increasing upwards. Stop the temperature increase at about 230°. Measure the difference on the graph between the initial and final mass-temperature plateaux, or mass-time plateaux, which corresponds to the loss of mass. The declared loss of mass for the certified reference material is stated on the label.

Procedure. Apply the same procedure to the substance to be examined, using the conditions prescribed in the monograph. Calculate the loss of mass of the substance to be examined

from the difference measured in the graph obtained. Express the loss of mass as per cent Δ *m/m*.If the apparatus is in frequent use; carry out temperature verification and calibration regularly. Otherwise, carry out such checks before each measurement.Since the test atmosphere is critical, the following parameters are noted for each measurement: pressure or flow rate, composition of the gas. Set the initial temperature, heating rate, and final temperature according to the manufacturer's instructions, and initiate the temperature increase. Alternatively, conduct an examination of the thermo-gram over a wide range of temperatures (typically, from room temperature to the decomposition temperature, or 10° to 20° above the melting point at a heating rate of 1° to 20° per minute). Calculate the mass gain or loss, expressing the change in mass as percentage.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a thermoanalytical technique that is used to demonstrate the energy phenomena, produced during heating or cooling of a substance (or a mixture of substances) and to determine the changes in enthalpy and specific heat and the temperatures at which these occur. The DSC instrument measures the heat flow in and out of both a sample and reference crucible during a controlled temperature programme. The sample crucible usually contains the substance (or a mixture of substances) under study and the reference crucible is either left empty or is loaded with an inert reference material relevant to the sample under investigation. Both the sample and reference are maintained at very nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. The reference sample should have a well defined heat capacity over the range of temperatures to be scanned.

Instrumentation. A typical DSC instrument consists of two sealed crucibles: a sample crucible and a reference crucible (which is generally an empty sample pan). A temperature-programming device, thermal detector(s) and a recording system, which can be connected to a computer, are attached. The measurements are carried out under a controlled atmosphere. Calibration of the apparatus for temperature and enthalpy change is done using Indium or Zinc of high purity or any other suitable certified material, according to the manufacturer's instructions.

Operating procedure. Weigh an appropriate quantity of the substance to be examined and place in the sample crucible. Set the initial and final temperatures, and the heating rate according to the operating conditions prescribed in the monograph. Begin the analysis and record the thermogram, with the temperature and/or time on the *x-axis* and the energy change on the *y-axis*. The temperature at which the phenomenon occurs (the onset temperature) corresponds to the intersection of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (Fig 2.4.31-1). The peak of the curve indicates the end of the thermal phenomenon. The enthalpy of the phenomenon is proportional to the area under the curve limited by the baseline; the proportionality factor is determined from the measurement of the heat of fusion of a known substance (e.g., Indium) under the same operating conditions. Each thermogram may be accompanied by the following data: conditions employed, record of last calibration, sample size and identification (including thermal history), container, atmosphere (identity, flow rate, pressure), direction and rate of temperature change, instrument and recorder sensitivity.

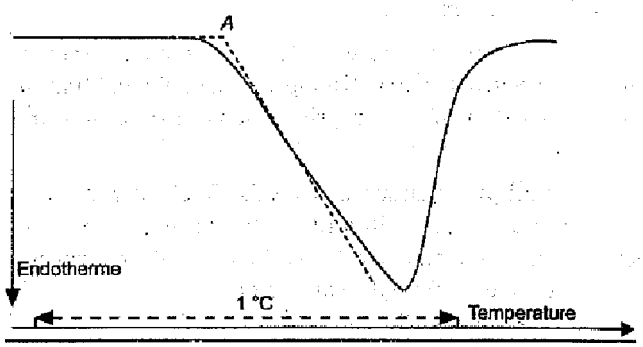


Fig.2.4.31-1: Endothermic thermogram

Applications. Basic principle underlying applicability of the DSC technique is that, when the sample undergoes a physical transformation such as phase transitions, more (or less) heat will need to flow to it than the reference to maintain both at the same temperature.

Phase changes. The typical examples of phase changes are:

- Solid - solid transition, *e.g.*, polymorphic phase transition, glass transition, desolvation, amorphous-crystalline
- Solid - liquid transition, *e.g.*, melting
- Solid - gas transition, *e.g.*, sublimation
- Liquid - solid transition, *e.g.*, freezing, recrystallisation
- Liquid - gas transition, *e.g.*, evaporation

These changes can be demonstrated as changes in enthalpy or heat capacity as a function of temperature.

Changes in chemical composition. Measurement of heat and temperatures of reaction under given experimental conditions may be done using DSC, *e.g.*, the kinetics of decomposition or the kinetics of desolvation.

Application to phase diagrams. Establishment of phase diagrams for solid mixtures can be achieved which is an important step in the preformulation and optimisation of the freeze-drying process.

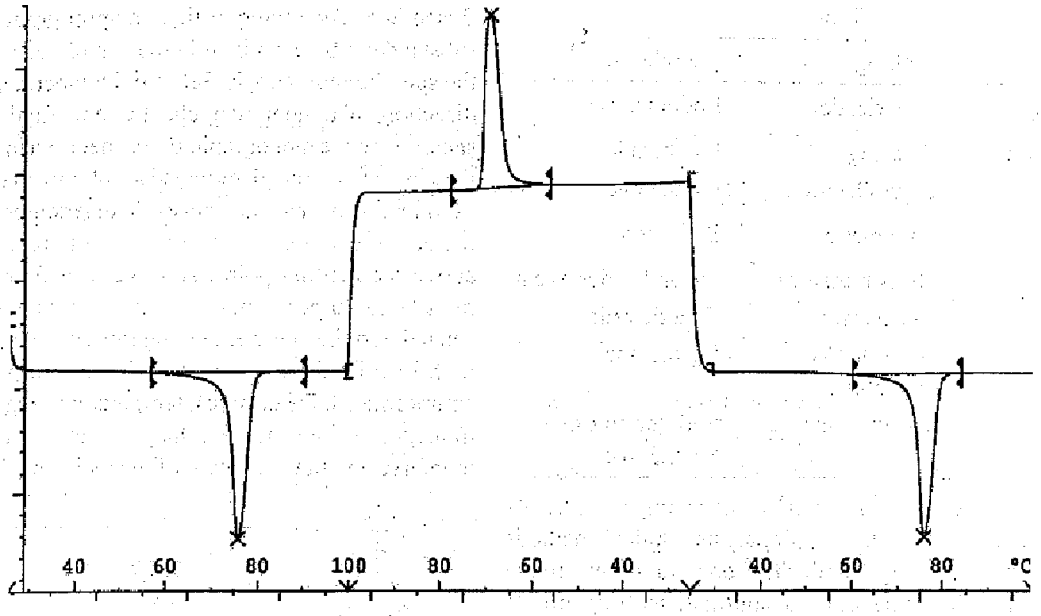


Fig. 2.4.31-2: Enantiotropic behaviour of ethambutol hydrochloride

Determination of purity. The measurement of the heat of fusion and the melting point by DSC enables to determine the purity content of a substance from a single thermal diagram, requiring only a few milligrams of sample with no need for repeated accurate measurements of the true temperature. The determination of the molar purity by DSC is based on the use of a mathematical approximation of the integrated form of the Van't Hoff equation applied to the concentrations (not the activities) in a binary system.

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times x_2$$

where, x_2 = mole fraction of the impurity i.e. the number of molecules of the impurity divided by the total number of molecules in the liquid phase (or molten phase) at temperature T (expressed in kelvins),

T_0 = melting point of the chemically pure substance, in kelvins,

ΔH_f = molar heat of fusion of the substance, in joules,

R = gas constant for ideal gases, in joules·kelvin^{-1}·mole^{-1}.

Detection and/or quantitation of stereoisomeric impurities. There are substances (*e.g.*, ethambutol hydrochloride) that show typical behaviour of polymorphic phase transformation, *viz*, one polymorphic form of the drug gets convert into the second form before melting point and the transformation is reversible when temperature is increased or decreased (Fig. 2.4.31-2). This kind of phenomenon is known as enantiotropic polymorphism. This solid-state property of the substances is sometime characteristic for individual stereoisomers. Enthalpy associated with the polymorphic phase transformation for individual stereoisomers can be used in qualitative, and also quantitative applications, as the same is directly proportional to the amount of substance under investigation.

Transition and Melting Point Temperatures

As a specimen is heated, transitions can be observed using differential scanning calorimetry (DSC), differential thermal analysis (DTA), or hot-stage microscopy. In heat-flux DSC, the heat differential between the sample and reference material is determined. In power compensation DSC, the sample and reference materials are maintained at the same temperature, using individual heating elements, and the difference in power input to the two heaters is recorded. DTA monitors the difference in temperatures between the sample and the reference. The transitions that may be observed include those shown in Table 1. In the case of melting, both an "onset" and a "peak" temperature can be determined objectively and reproducibly, often to within a few tenths of a degree. Although these temperatures are useful for characterizing substances, and the difference between the two temperatures is indicative of purity, the values cannot be directly compared to visual "melting-range" or "melting-point" values or with constants such as the triple point of the pure material.

Table 1

Solid to liquid	Melting	Endothermic
Liquid to gas	Vaporization	Endothermic
Liquid to solid	Freezing	Exothermic
	Crystallization	Exothermic
Solid to gas	Sublimation	Endothermic
Solid to solid	Glass transition	Second order event
	Desolvation	Endothermic
	Amorphous to crystalline	Exothermic
	Polymorphic	Endothermic or Exothermic

Furthermore, caution should be used when comparing results obtained by different methods of analysis. Optical methods may measure the melting point as the temperature where the last trace of solid coalesces. In contrast, melting points measured by DSC may refer to the onset temperature or the temperature where the maximum melting rate (vertex) was observed. However, the vertex is sensitive to sample weight, heating rate, and other factors, whereas the onset temperature is less affected by these factors. With thermal techniques, it is necessary to consider the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

Reporting Results of Instrumental Methods. A complete description of the conditions employed should accompany each thermo gram, including make and model of instrument; record of last calibration; specimen size and identification (including previous thermal history); container; identity, flow rate, and pressure of gaseous atmosphere; direction and rate of temperature change, and instrument and recorder sensitivity.

Determination of Transition Temperature (Melt Onset Temperature) and Melting Point Temperature

Apparatus. Use DTA or DSC instrumentation equipped with a temperature-programming device, thermal detector (s), and a recording system that can be connected to a computer, unless otherwise prescribed by the specific monograph for which this chapter is being used.

Calibration. Calibrate instrumentation for temperature and enthalpy changes, using indium or another suitable certified material. Temperature calibration is performed by heating a standard through the melting transition and comparing the extrapolated onset of melting point of the standard to the certified onset of melting point. The temperature calibration should be conducted at the same heating rate as the experiment. Enthalpy calibration is performed by heating a standard through the melting transition and comparing the calculated heat of fusion to the theoretical value.

Procedure. Accurately weigh an appropriate quantity of the substance to be examined in the sample pan, as described in the specific monograph. Set initial temperature, heating rate, direction of temperature change, and final temperature as specified in the monograph. If not specified in the monograph, these parameters are determined as follows: make a preliminary examination over a wide range of temperatures (typically, room temperature to decomposition temperature, or about 10 to 20 above the melting point) and over a wide range of heating rates (1 to 20 per minute) to reveal any unexpected effects. Then determine a lower heating rate such that decomposition is minimized and the transition temperature is not compromised. Determine a temperature range bracketing the transition of interest such that the baseline can be extended to intersect with the tangent of the melt (see Fig. 2.4.31-3).

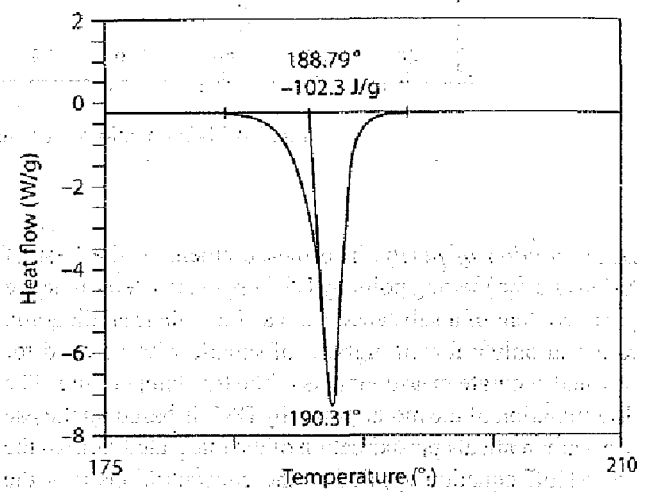


Fig. 2. 4.31-3: Thermo-gram.

In examining pure crystalline materials, rates as low as 1° per minute may be appropriate, whereas rates of up to 20° per minute are more appropriate for polymeric and other semi crystalline materials. Begin the analysis, and record the differential thermal analysis curve with the temperature on the *x*-axis and the energy change on the *y*-axis. The melting temperature (melt onset temperature) is the intersection (188.79°) of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (see Fig. 1). The vertex is the temperature at the peak of the curve (190.31°). The enthalpy of the event is proportional to the area under the curve after application of a baseline correction

Thermo-Microscopy

Thermo-microscopy is an analytical technique that involves monitoring Phase changes of the sample in polarized light using a microscope as a function of temperature. It is useful to confirm transitions such as melts, re-crystallizations, and solid-state transformations using a visual technique. The

observations made in thermo-microscopy allow the nature of the phenomena detected using thermo-gravimetry and differential thermal analysis to be clearly identified.

Apparatus. The apparatus consists of a microscope fitted with a light polarizer, a hot plate, a temperature and heating rate and/or cooling rate programmer and a recording system for the transition temperatures. A video camera and video recorder may be added.

Eutectic Impurity Analysis

Melting transition for an absolutely pure crystalline compound should occur within an infinitely narrow range. A broadening of the melting range, due to impurities, provides a sensitive criterion of purity. The effect is apparent visually by examination of thermo-grams of substance differing by a few tenths percent in impurity content. The basis of any calorimetric purity method is the relationship between the melting and freezing point depression and the level of impurity. The melting of a compound is characterized by the absorption of latent heat of fusion, H_f, at a specific temperature, T_o. A substance that is 99 per cent pure is about 20 per cent molten at 3° below the melting point of the pure substance The parameters of melting (melting range, H_f, and calculated eutectic purity) are readily obtained from the thermo-gram of a single melting event using a small test substance, and the method does not require multiple, precise actual temperature measurements. Thermo-gram units are directly convertible to heat transfer, millicalories per second. Fig.2.4.31-4. explain the effect of impurities on melting.

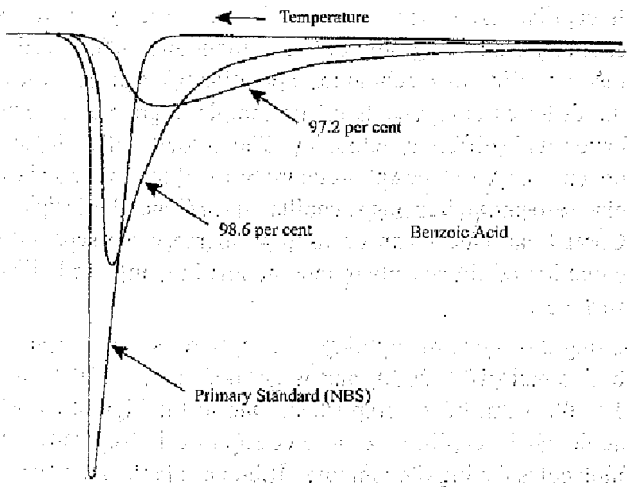


Fig. 2.4.31-4: Superimposed thermo-grams illustrating the effect of impurities on melting peak shape

The lowering of the freezing point in dilute solutions by molecules of nearly equal size is expressed by a modified van't Hoff equation:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f} \times (K_D - 1) \qquad \text{-----(1)}$$

Where, T = absolute temperature in kelvins,
X_2 = mole fraction of minor component (solute, impurity),
H_f = molar heat of fusion of the major component in Joules per mol,
R = gas constant in Joules per mol × kelvins,
K_D = distribution ratio of solute between the solid and liquid phases.

Assuming that the temperature range is small and that no solid solutions are formed ($K_D = 0$).Integration of the van't Hoff equation yields the following relationship between the mole fraction of impurity and the melting-point depression:

$$X_2 = \frac{(T_o - T_m)\Delta H_f}{RT_o^2} \qquad \text{------(2)}$$

Where, T_o = melting point of the pure compound, in kelvins,
T_m = melting point of the test specimen, in kelvins.

With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed test specimen temperature, T_s, versus the reciprocal of the fraction melted, 1/F, at temperature T_s, should yield a straight line with the slope equal to the melting-point depression ($T_o - T_m$). The theoretical melting point of the pure compound is obtained by extrapolation to 1/F = 0:

$$T_S = T_o - \frac{RT_o^2 X_2 (1/F)}{\Delta H_f} \qquad \text{-----(3)}$$

Substituting the experimentally obtained values for T_o, T_m, H_f, and T_o in equation (2) yields the mole fraction of the total eutectic impurity, which, when multiplied by 100, gives the mole-percentage of total eutectic impurities.

Deviations from the theoretical linear plot also may be due to solid solution formation (K_D 0), so that care must be taken in interpreting the data.

To observe the linear effect of the impurity concentration on the melting-point depression, the impurity must be soluble in the liquid phase or melt of the compound, but insoluble in the solid phase, i.e., no solid solutions are formed. Some chemical similarities are necessary for solubility in the melt. For example, the presence of ionic compounds in neutral organic compounds and the occurrence of thermal decomposition may not be reflected in purity estimates. The extent of these theoretical limitations has been only partially explored.

Impurities present from the synthetic route often are similar to the end product, hence there usually is no problem of solubility in the melt. Impurities consisting of molecules of the same shape, size, and character as those of the major component can fit into the matrix of the major component without disruption of the lattice, forming solid solutions or inclusions; such impurities are not detectable by DSC. Purity estimates are too high in such cases. This is more common with less-ordered crystals as indicated by low heats of fusion.

In addition, the method is reliable when the purity of the major component is greater than 98.5 mol percent and the materials are not decomposed during the melting phase.

Impurity levels calculated from thermo-grams are reproducible and generally reliable within 0.1percent for ideal compounds. Compounds that exist in polymorphic form cannot be used in purity determination unless the compound is completely converted to one form. On the other hand, DSC and DTA are inherently useful for detecting, and therefore monitoring, polymorphism.

Procedure. The actual procedure the calculations to be employed for eutectic impurity analysis are dependent on the particular instrument used. Consult the manufacturer's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

2.4.32. Capillary Electrophoresis

Capillary Electrophoresis is a method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

Apparatus

An apparatus for capillary electrophoresis consists of:

a) a high-voltage, controllable direct-current power supply;

b) buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;

c) electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply;

d) a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;

e) a suitable injection system;

f) a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds;

g) a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility;

h) a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

Capillary Zone Electrophoresis

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small (M_r < 2000) and large molecules (2000 < M_r < 100 000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimisation

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main

factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage. A Joule heating plot is useful in optimising the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity. Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

Temperature. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolytic solution parameters

Buffer type and concentration. Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

Buffer pH. The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents. Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

Additives for chiral separations. For the separation of optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (a-, b-, or g-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionisable (aminomethyl, carboxymethyl, sulphobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

Capillary Gel Electrophoresis

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

Characteristics of gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulphate and the samples are denatured by heating in a mixture of sodium dodecyl sulphate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

Capillary Isoelectric Focusing

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilisation.

Loading step. Two methods may be employed:

a) loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;

b) sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

Focusing step. When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

Mobilisation step. If mobilisation is required for detection, use one of the following methods.

a) Mobilisation is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;

b) Mobilisation is accomplished by applying positive pressure after the focusing step;

c) Mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilised in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \times \sqrt{\frac{D\,(dpH/dx)}{E\,(-d\mu/dpH)}}$$

Optimisation

The main parameters to be considered in the development of separations are:

Voltage. Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary. The electro-osmotic flow must be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions. The anode buffer reservoir is filled with a solution with a pH lower than the pH of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pH of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

2.4.33. Isoelectric Focusing

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

General aspects

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. haemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

Apparatus

a) a controllable generator for constant potential, current and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended; a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;

b) a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

Procedure

Preparation of the gels

Mould The mould (see Figure 2.4.33-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

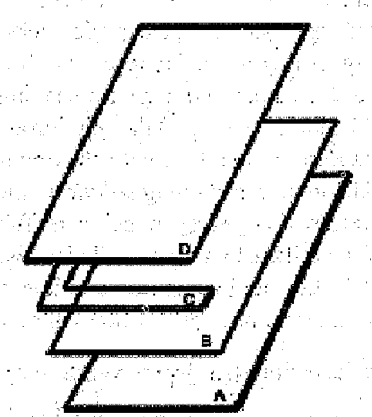


Fig. 2.4.33-1–Mould

7.5 per cent polyacrylamide gel. Dissolve 29.1 g of *acrylamide* and 0.9 g of *methylenebisacrylamide* in 100 ml of *water*. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water*. Mix carefully and degas the solution.

Preparation of the mould. Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g per litre solution of *ammonium persulphate* and 0.25 volumes of *tetramethyl-ethylenediamine*. Immediately fill the space between the glass plates of the mould with the solution.

Method

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in fixing solution for isoelectric focusing in *polyacrylamide gel*. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution and add 200 ml of *destaining solution* . Incubate with shaking for 1 hour. Drain the gel, add *coomassie staining solution* . Incubate for 30 minutes. Destain the gel by passive diffusion with *destaining solution* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

Variations to the Procedure (subject to validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

a) the use of commercially available pre-cast gels and of commercial staining and destaining kits,

b) the use of immobilised pH gradients,

c) the use of rod gels,

d) the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,

e) variations in the sample application procedure, including different sample volumes or the

f) use of sample application masks or wicks other than paper,

g) the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,

h) the inclusion of a pre-focusing step, the use of automated instrumentation,

i) the use of agarose gels.

Specified Variations to the General Method

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

a) the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;

b) the use of alternative staining methods;

c) the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and

d) the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

Points to Consider

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

2.4.34. Nuclear Magnetic Resonance Spectrometry

Nuclear Magnetic Resonance is based on absorption of radio-waves of certain nuclei in strong Magnetic fields.

Nuclear Magnetic Resonance (NMR) Spectrometry is an analytical method in particular suitable for the elucidation of the chemical structure of organic molecules by means of interpretation of their NMR spectra, arising from, for example, ^{1}H or the X-nuclei ^{13}C, ^{19}F, ^{15}N, ^{31}P. The spectra can be used for qualitative and quantitative purposes. Under suitable experimental conditions, the integrated NMR intensities of the signals are directly proportional to the number of nuclear spins of the molecular group responsible for the signal. These integrals can be used for quantitative analysis.

Principle

Placing an ensemble of nuclei with angular momentum and a magnetic moment in a static magnetic field (B_0) causes the nuclei to arrange themselves in different, quantum-mechanically controlled orientations in relation to the axis of the magnetic field. These orientations are different in energy. An oscillating high-frequency magnetic field (B_1), perpendicular to B_0, will cause transitions between these orientations with net energy absorption. According to the resonance condition $\omega_0 = \gamma B_0$ (γ = gyromagnetic ratio, ω_0= Larmor frequency), either the B_0 magnetic field or the frequency (ω_1) of the B_1 field may be varied to achieve a spectrum. The coherent radiation emitted during the return to the initial state is observed in the form of a decay curve, called the free induction decay (FID). Subsequent Fourier transformation gives the spectrum in the frequency domain, providing information about the molecular structure. Additional radiofrequency fields may be applied during acquisition of the FID signal to suppress scalar (through-bond) interactions between nuclei (called 'decoupling'). One and multi-dimensional techniques can be applied for qualitative and quantitative purposes, on samples in either the liquid or the solid state.

Important structural information is derived from the following spectroscopic features

Resonance frequency	Kind of nuclei observed
Number of resonance signals (siglets, multiplets)	Number of chemically distinct groups of nuclei
Chemicals shift δ (ppm)	Chemical nature and environment of the structural group observed
Intensity of resonance signals	Relative number of resonant nuclei per chemically distinct group
Multiplicity of coupling pattern	Number of nuclei that are scalar coupled to the observed nucleus
Coupling constant J(Hz)	Number of bonds in the coupling pathway , and its geometry

Correlations of different spectral parameters (e.g. chemical shift and coupling constant, or chemical shifts of different nuclei within one molecular system) can be performed by homo and hetero-nuclear two and higher dimensional methods. Information about the relaxation times T_1 and T_2, nuclear overhauser effects (NOEs) and the kinetics of time-dependent processes is also accessible from appropriate experiments.

Apparatus

A high-resolution NMR spectrometer consists of at least the following parts:

- a magnet to deliver the constant magnetic field B_0;
- a temperature-controlled probe to contain the sample, to deliver the radiofrequency pulse and to detect radiation emitted by the sample;
- an electronic console to generate high-power radiofrequency pulses and to collect and digitise the FID signal; this unit also maintains the stability of the instrument electronics;
- a data acquisition and processing unit (computer); and may also include:
- a continuous flow cell for coupled liquid chromatographic-NMR or flow injection analysis;
- a system for pulsed field gradient NMR.

The high magnetic field is generated by a superconducting coil in a Dewar flask filled with liquid helium. The probe typically contains the sample in a 5 mm-outer-diameter sample tube or in a flow cell, and is connected to the electronics cabinet by RF cables carrying lock, ^{1}H-, and X-nucleus frequencies. Additional devices for tuning and matching the electronic circuits are essential, and sample temperature control is often used.

The NMR spectrometer should be demonstrated to be operating correctly. Appropriate tests to demonstrate this are, typically, measurement of linewidths at half height for defined peaks under defined acquisition conditions, signal-to-noise ratios (S/N) for standard mixtures, pulse power (measured as a 90° pulse width), and pulse reproducibility. All instrument manufacturers publish specifications and measurement protocols for these parameters for specific instrument/probe combinations, and compliance with these specifications should be demonstrated.

Fourier Transform NMR (FT-NMR)

Contemporary spectrometers generally operate according to the Fourier transform (FT) principle: after exciting the sample with a radiofrequency pulse of appropriate frequency (υ),

amplitude (B_1) and duration (tp) and a succeeding short dead time (td) (to enable the electronics to recover), the amplified analogue FID signal is sampled during the acquisition time (tac) and digitised with an analogue-to-digital converter (ADC), and the results are stored in the spectrometer memory. The receiver output is amplified prior to digitisation to maximise sensitivity without saturating the ADC. In case of observation of X-nuclei, the standard experiment includes, if necessary, broadband 1H decoupling, i.e. irradiation of all the protons during the experiment. To increase the S/N, multiple FID signals may be accumulated coherently and summed. Fourier transformation of this time-domain data gives the frequency-domain spectrum.

Parameters

The following acquisition parameters influence the result of an FT experiment, and should be adjusted and controlled.

Pulse width (ôp)

The excitation pulse is directed along the x-axis of the so-called rotating frame, its duration (or 'width', ôp) determines the flip angle (θ) and thus the intensity (I) of the resonance signal:

$$\theta = \gamma \times B_1 \times \tau_p \qquad \text{------(1)}$$

$$M_y = M_o \times \sin\theta \qquad \text{------(2)}$$

The observed magnetisation M_y is maximum at θ = 90°. The pulse duration should be short to guarantee that all signals in the spectral width (SW) are excited to a similar degree. The magnetisation decays due to relaxation processes.

Dead time (t_d)

The dead time is the time between the end of the pulse and start of the acquisition, it is necessary for technical reasons and care should be taken as it may influence signal intensities and peak phase. Rapidly decaying signals (giving rise to broad spectral lines) are reduced in intensity by more than slowly decaying signals (which give rise to narrow spectral lines).

Acquisition time (t_{ac})

The acquisition time (t_{ac}) is related to the spectral width (i.e. the whole observed region) and the number of digital data points (DP) collected during signal acquisition:

$$t_{ac} = \frac{DP}{2SW} \qquad \text{------(3)}$$

Maximal signal intensity and signal-to-noise ratio will be achieved if tac = 1.2/(πυ 1/2), where υ1/2 is the full width at half-height (fwhh), but it should be set to greater than 5/ (πυ 1/2) to minimize signal distortion.

Repetition time (t_r)

The spin-lattice relaxation (T_1) governs the time required for the spin system to return to equilibrium after a pulse. Relaxation can be reduced by the use of special reagents. For quantitative purposes, the repetition time used should be set relative to T_1 and θ to avoid saturation effects.

Receiver gain

The analogue signal detected by the probe is amplified prior to digitisation and storage. The amplification, or receiver gain, should be set, either automatically or manually, so that the signal does not overload the ADC, which causes signal distortion, but allows random noise generated in the probe to be digitized (i.e. is non-zero).

Optimization of acquisition quantitative purposes

Besides the acquisition parameters, signal intensity is also influenced by several processing parameters. After collecting a sufficient number of scans, the resulting FID is Fourier transformed. For reliable quantitative purposes the following parameters have to be optimized.

Signal-to-Noise ratio (S/N)

This is the ratio between the intensities (as peak height) of a specified signal in the nuclear magnetic resonance spectrum and the random fluctuations in that signal, which is usually measured in a region of the spectrum that contains no signals from the analyte. A poor signal-to-noise ratio (S/N) limits the accuracy of peak integrations and quantitative analyses. An S/N equal to or greater than 150:1 allows peak integrations with a standard deviation of less than 1 per cent. Contemporary spectrometers have software algorithms to report the S/N of appropriate peaks. A sufficiently high S/N can be difficult to obtain when analysing dilute solutions, and especially when detecting nuclei other than ^1H. Methods to enhance the S/N include:

- increasing the number of accumulated scans (n), as S/N increases with $\sqrt{n}$;
- use of exponentional multiplication on the FID signal before Fourier transformation; the exponentional multiplication factor should be in the order of the peak full width at half-height (*fwhh*);
- use of spectrometers with a higher magnetic field B_o, since S/N is proportional to $B_o^{3/2}$;
- use of digital filtering to reduce noise;
- use of probes that maximize the filling factor;
- use of cryoprobes that reduce thermal noise.

Integration region

The intensity of the nuclear magnetic resonance signals is obtained by a quasi- analogue signal integration either by a

stepped-line plot or, more accurately, by separate line integration and digital data presentation. In liquid state, nuclear magnetic resonance signals have Lorentzian line shape. Unless otherwise specified in the monograph or when peak overlap occurs, the same integration range, expressed as a multiple of the peak fwhh, should be used for the monitored peak and the reference peak.

Dynamic range

The dynamic range of the analogue-to-digital converter (ADC) determines the minimum intensity line that can be observed or quantified when integrating 2 signals with the same line width in a spectrum. A 16-bit ADC allows identification of a signal of 0.003 per cent intensity relative to a strong signal completely filling the dynamic range of the ADC.

NMR of samples in solution

Most nuclear magnetic resonance experiments are performed on dilute solutions (about 1 per cent) of the analyte in an appropriate solvent, which can be spiked with a suitable standard for chemical shift calibration.

Solvents

The solvent should be able to dissolve the analyte without further interaction if not otherwise intended. To minimise the intense solvent signals, fully deuterated solvents (*deuterium oxide, deuterated chloroform, deuterated dimethyl sulfoxide, deuterated acetone, deuterated methanol, etc.*) should be used. The solvent atoms give signals that are easily identified by their chemical shift and can be used to calibrate the chemical shift axis (secondary reference).

Referencing

The spectral feature most dependent on the chemical environment of the atom in the molecule is the chemical shift, designated as d and reported in parts per million. The chemical shift between the resonance for an nuclear magnetic resonance active nucleus X ($\delta_{X,sample}$) is measured in parts per million as the difference between the resonance frequency of that nucleus ($\upsilon_{X,sample}$) and that of an internal shift reference standard ($\upsilon_{X,reference}$), both in hertz, divided by the basic spectrometer operating frequency ($\upsilon_{X,reference}$), in megahertz, at a given B_0.

$$\delta_{X,sample} = \frac{(\upsilon_{X,sample} - \upsilon_{X,reference})}{\upsilon_{X,reference}} \quad \text{------ (4)}$$

By convention, the standard for exact chemical shift referencing is the ^1H resonance of *tetramethylsilane* (TMS), setting $\delta_{TMS} = 0$ ppm. Formally, once the ^1H shift scale has been referenced relative to TMS, the exact frequency of any other X resonance can be calculated and its chemical shift scale calibrated. The frequency of a (secondary) reference standard at $\delta_X = 0$ ppm ($\upsilon_{X,reference}$) is calculated from the ^1H frequency of TMS ($\upsilon_{H,TMS}$) and a tabulated value of the ratio ($\Xi_{X,reference}$) of the isotope-specific frequency in relation to that of ^1H in TMS:

$$\upsilon_{X,reference} = \upsilon_{H,TMS} \times \Xi_{X,reference} \quad \text{------ (5)}$$

Reference standards at $\delta_X = 0$ ppm and corresponding $\Xi_{X,reference}$ values are shown, below:

Nucleus	Water	$\Xi_{X,reference}$	Other Solvents	$\Xi_{X,reference}$
^1H	DSS[b]	1.00000000	TMS	1.00000000
^{13}C	DSS[b]	0.25144953	TMS	0.25145020
^{15}N	NH_3	0.10132912	CH_3NO_2	0.10136767
^{19}F	CF_3COOH	Not reported	CCl_3F	0.94094011
^{31}P	H_3PO_4 (85 per cent)	0.40480742	$(CH_3O)_3PO$	0.40480864

[a]chemical shift depends on pH

[b]DSS = sodium 2,2-dimethyle-2-silapentane-5-sulfonate

In practice, X chemical shifts are referenced directly using an appropriate standard. In ^1H and ^{13}C nuclear magnetic resonance, internal referencing is mainly used, where the reference is added directly to the system under study. In ^{15}N, ^{19}F and ^{31}P nuclear magnetic resonance, external referencing is often suitable, involving sample and reference contained separately in coaxial cylindrical sample tubes.

Lock

In order to prevent drifting of the spectrum over time, a stabilising procedure, called field-frequency locking, is performed. The ^2H (deuterium) signal arising from deuterated solvents is used to achieve this, unless otherwise specified in the monograph.

Qualitative Analysis

The principal use for qualitative nuclear magnetic resonance spectra is as an identity test, in which the ^1H or ^{13}C spectrum of a test sample is compared to the spectrum of a reference sample or, less commonly, with a published reference spectrum. Spectra of reference and test samples should be acquired using the same procedure and operational conditions. The peaks in the 2 spectra, or characteristic regions of the spectra, should correspond in position, intensity and multiplicity. In appropriate cases, mathematical comparison, such as calculation of a correlation coefficient, may be appropriate. In the absence of a reference standard, an in-house reference may be used, whose identity has been demonstrated by alternative methods, or the demonstration that the NMR spectrum is fully consistent with the reported structure for that material.

Quantitative Analysis

Signal intensity in the basic nuclear magnetic resonance experiment is the integrated area under the signal curve measured. Only when 2 signals have equal fwhh and the same multiplicity may signal height serve as a measure of intensity. Under conditions of essentially complete relaxation between scans, the signal intensity (I_A) is a true measure of the number (N_A) of nuclei responsible for the respective signal:

$$I_A = K_S \times N_A \qquad \text{------ (6)}$$

The constant K_S includes fundamental constants, properties of the sample and receiver parameters, and can be omitted in cases where signal intensities are compared, giving the direct relation between the numbers of nuclei in the 2 compared structure groups A and B

$$\frac{I_A}{I_B} = \frac{N_A}{N_B} \qquad \text{------ (7)}$$

The numbers (N_i) of nuclei belonging to different structure groups of 1 molecule are small integers. The values measured are rounded to the closest integer numbers. However, the proper operation of acquisition and processing of the spectrometer is easily checked by comparing exact intensities within a spectrum of any suitable organic compound of known structure.

In addition to the fact that the intensities of signals arising from each component in a mixture are related to each other by small integer numbers, the relative molar amounts of these components can be measured by comparison of the normalized intensities of resonances from different components. The molar ratio of 2 components of a mixture is calculated according to the following equation

$$\frac{n_A}{n_B} = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \qquad \text{------ (8)}$$

The determination is only valid in cases where the structure of the molecules for which I_A and I_B are determined are known (or at least the values of N for the monitored groups). Determinations are made using either an internal standard method or a peak-normalization procedure.

Internal standard method

The mass (m_A) of an analyte (A) can be determined if a known mass (m_B) of a substance (B) with a known percentage content (P_B) is added to the solution as an intensity standard. Equation (8) can be converted to equation (9)

$$m_A = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \times \frac{M_A}{M_B} \times m_B \times \frac{P_B}{100} \qquad \text{------ (9)}$$

Here, *Mi* are the molecular masses.

The intensity standard has to be carefully chosen; it should be completely soluble in the solvent and should produce only a small number of signals, and the 'monitor group' should have a signal in an empty spectral region. A compound of high purity and with a relatively high molecular mass is recommended for this purpose.

Normalisation procedure

The relative proportions of components in a mixture, the degree of substitution in a structurally modified polymer, or the amount of a contaminant can be determined by comparison of the relative intensities of resonances present.

The experimental method should be validated to ensure that there is no overlap of the relevant signals. When the contaminant is of poorly defined structure or molecular mass (e.g. an emulsifier), addition of known amounts of that material to the nuclear magnetic resonance tube will allow a calibration curve to be constructed.

Method

Liquid Sample handling

Dissolve the sample in the solvent to which the appropriate reference material may have been added to calibrate chemical shift, as prescribed in the monograph. For quantitative analysis, the solutions must be free from solid particles. Some quantitative analyses may require an internal standard to be included, so that integrations of resonances from the test sample and the reference material can be compared. Appropriate references and concentrations are indicated in the specific monographs. In other quantitative analyses, the result is obtained by comparing the relative intensities of 2 or all of the resonances that arise from the test sample. After loading the sample into a tube and capping, the sample is introduced into the nuclear magnetic resonance magnet, the experimental parameters are loaded and the experiment is executed. Key experimental parameters are indicated in the monograph.

Measurement

Equilibrate the sample in the probe, and optimize the instrument to achieve best resonance conditions and to maximize the S/N by tuning and matching the probe, and make adjustments to maximize magnetic field homogeneity over the sample volume (called 'shimming'). Record, or save to computer, the parameter settings. An experiment may be composed of multiple pulse-acquisition-delay sequences, and the individual FIDs are summed in the computer memory, with random noise being averaged out. When an appropriate S/N has been achieved, the FID is stored and the frequency-domain spectrum is generated by Fourier transformation of the summed FIDs.

Nuclear magnetic resonance in the solid state

Samples in the solid state can be analysed using nuclear magnetic resonance spectrometers specially equipped for that purpose. Certain technical procedures make observable individual lines for individual atomic sites with a valuable extension of the applicability of nuclear magnetic resonance to inorganic materials as well.

One technique is the rapid rotation (4-30 kHz) of the powdered sample in a rotor (about 4 mm outer diameter) inclined at an angle of 54.7° (the 'magic angle') to the direction of the B_0 magnetic field axis. This technique is named magic angle spinning (MAS). Another effective tool is high-power decoupling and a 3rd method is the transfer of polarisation from easily excitable nuclei towards less-polarisable nuclei, i.e. cross polarisation (CP). The combination of these techniques makes available high-resolution spectra containing much information about chemical and structural details in solid glassy, amorphous, and crystalline materials of ceramic, polymeric or mineralogical origin.

If nuclear magnetic resonance is applied to a solid state samples, full details of the procedure are provided in the specified monograph.

2.4.35. Bulk Density and Tapped Density of Powders

Bulk Density

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per ml (g/ml) although the international unit is kilograms per cubic meter (1 g/ml = 1000 kg/m³) because the measurements are made using cylinders. It may also be expressed in grams per cubic centimeter (g/cm³). The bulking properties of a powder are dependent upon the preparation, treatment, and storage of the sample, i.e., how it was handled. The particles can be packed to have a range of bulk densities; however, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results; it is essential to specify how the determination was made. The bulk density of a powder is determined by either measuring the volume of a known weight of powder sample, that may have been passed through a sieve, into a graduated cylinder (Method I), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (*Method II*) or a measuring vessel (*Method III*).

Method I and *Method III* are favored.

Method I. Measurement in a Graduated Cylinder

Procedure. Pass a quantity of material sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry graduated 250-ml cylinder (readable to 2 ml), gently introduce, without compacting, approximately 100 g of test sample (*M*) weighed with 0.1 per cent accuracy. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume (V_0) to the nearest graduated unit. Calculate the bulk density in g per ml by the formula m/V_0. Generally, replicate determinations are desirable for the determination of this property. If the powder density is too low or too high, such that the test sample has an untapped apparent volume of either more than 250 ml or less than 150 ml, it is not possible to use 100 g of powder sample. Therefore, a different amount of powder has to be selected as the test sample, such that its untapped apparent volume is 150 ml to 250 ml (apparent volume greater than or equal to 60 per cent of the total volume of the cylinder); the weight of the test sample is specified in the expression of results. For test samples having an apparent volume between 50 ml and 100 ml, a 100 ml cylinder readable to 1 ml can be used; the volume of the cylinder is specified in the expression of results.

Method II. Measurement in a Volumeter

Apparatus. The apparatus (Fig. 2.4.35-1) consists of a top funnel fitted with a 1.0-mm sieve. The funnel is mounted over

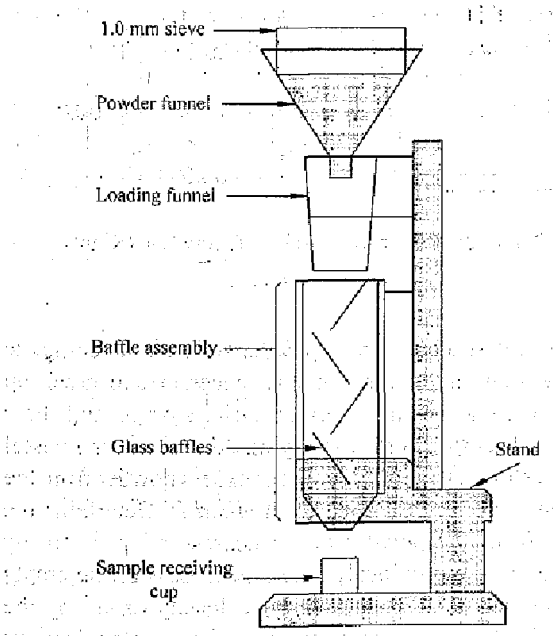


Fig. 2.4.35-1: Volumeter

a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup of specified capacity mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 ml volume with an inside diameter of 30.00 ± 2.00 mm) or a square (16.39 ± 0.2 ml volume with inside dimensions of 25.4 ± 0.076 mm).

Procedure. Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm^3 of powder with the square cup and 35 cm^3 of powder with the cylindrical cup. Carefully scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the sides of the cup, and determine the weight, M, of the powder to the nearest 0.1 per cent. Calculate the bulk density, in g/ml, by the formula:

$$\frac{M}{V_0}$$

in which V_0 is the volume, in ml, of the cup. Record the average of three determinations using three different powder samples.

Method III. Measurement in a Vessel

Apparatus. The apparatus consists of a 100-ml cylindrical vessel of stainless steel with dimensions as specified in Fig. 2.4.35-2.

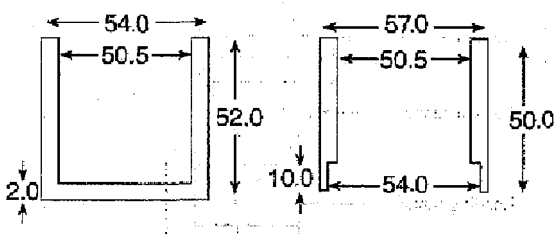


Fig. 2.4.35-2: Measuring Vessel (Left) and Cap (Right)

Dimension in millimetres

Procedure. Pass a quantity of powder sufficient to complete the test through a 1.0 mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrap the excess powder from the top of the vessel as described for *Method II*. Determine the weight (M_0) of the powder to the nearest 0.1 per cent by subtraction of the previously determined mass of the empty measuring vessel. Calculate the bulk density (g/ml) by the formula $M_0/100$, and record the average of three determinations using three different powder samples.

Tapped Density

The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample. Tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing a powder sample. After observing the initial powder volume or weight, the measuring cylinder or vessel is mechanically tapped, and volume or weight readings are taken until little further volume or weight change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop under its own weight a specified distance by either of three methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimize any possible separation of the mass during tapping down.

Method I

Apparatus. The apparatus (Fig. 2.4.35-3) consists of the following:

- A 250-ml graduated cylinder (readable to 2 ml with a mass of 220 ± 44 g
- A settling apparatus capable of producing, in 1 minute, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.

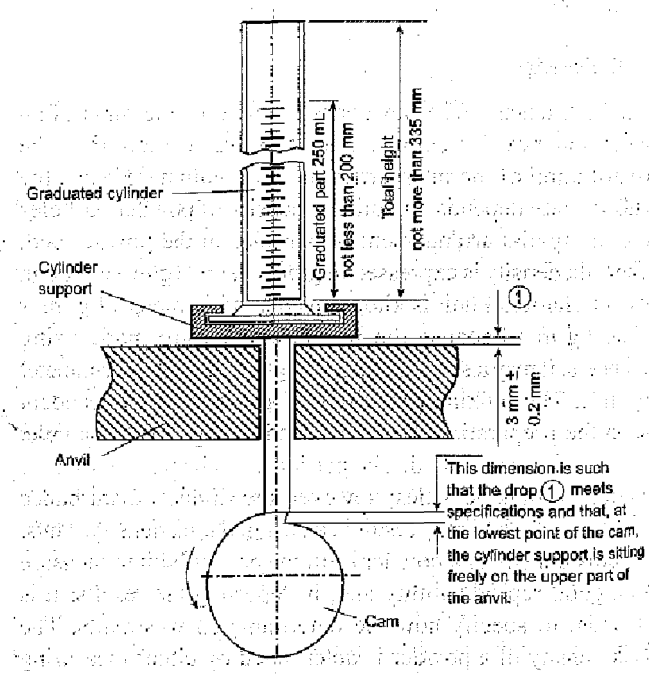

Graduated cylinder
Cylinder support
Graduated part 250 mL
not less than 200 mm
Total height
not more than 335 mm
①
3 mm +/- 0.2 mm
Anvil
This dimension is such that the drop ① meets specifications and that, at the lowest point of the cam, the cylinder support is sitting freely on the upper part of the anvil.
Cam

Fig. 2.4.35-3: Settling device for Powder Samples

Dimension in millimetres

Procedure. Proceed as described above for the determination of the bulk volume (V_0). Secure the cylinder in the holder

Carry out 10, 500, and 1250 taps on the same powder sample and read the corresponding volumes V_{10}, V_{500}, and V_{1250} to the nearest graduated unit. If the difference between V_{500} and V_{1250} is less than 2 ml, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 2 ml, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 2 ml. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density (g/ml) using the formula m/V_F in which V_F is the final tapped volume. Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results. If it is not possible to use a 100 g test sample, use a reduced amount and a suitable 100 ml graduated cylinder (readable to 1 ml) weighing 130 ± 16 g and mounted on a holder weighing 240 ± 12 g. The modified test conditions are specified in the expression of the results.

Method II

Apparatus and Procedure. Proceed as directed under *Method I* except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per minute.

Method III

Apparatus and Procedure. Proceed as directed in *Method III—Measurement in a Vessel* for measuring bulk density using the measuring vessel equipped with the cap shown in Figure 2. The measuring vessel with the cap is lifted 50–60 times per minute by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap, and carefully scrap excess powder from the top of the measuring vessel as described in Method III—Measurement in a Vessel for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the two masses obtained after 200 and 400 taps exceeds 2 per cent, carry out a test using 200 additional taps until the difference between succeeding measurements is less than 2 per cent. Calculate the tapped density (g/ml) using the formula $M_P/100$ where M_P is the mass of powder in the measuring vessel. Record the average of three determinations using three different powder samples. The test conditions including tapping height are specified in the expression of the results.

Measures of Powder Compressibility

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the *Compressibility Index* or the *Hausner Ratio* as described below.

The *Compressibility Index* and *Hausner Ratio* are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the *Compressibility Index* and the *Hausner Ratio*.

Compressibility Index. Calculate by the formula:

$$\frac{100(V_0 - V_F)}{V_0}$$

Where, V_0 = unsettled apparent volume
V_F = final tapped volume

Hausner Ratio

$$\frac{V_0}{V_F}$$

Depending on the material, the compressibility index can be determined using V_{10} instead of V_0. If V_{10} is used, it is clearly stated with the results.

2.4.36. Completeness of Solution

Procedure. Place the quantity of the substance specified in the individual monograph in a meticulously cleansed, glass-stoppered, 10 ml glass cylinder approximately 13 mm × 125 mm in size. Using the solvent that is specified in the monograph or on the label of the product, fill the cylinder almost to the constriction at the neck. Shake gently to effect solution: the solution is not less clear than an equal volume of the same solvent contained in a similar vessel and examined similarly.

2.4.37. Crystallinity

This chapter provides general information on crystallinity and refers to the various techniques described.

The Concept of Crystallinity

Most organic and inorganic compounds of pharmaceutical relevance exist as a solid material, which can be characterized by a structure located between a perfectly ordered crystal and an amorphous material.

Real crystals lie somewhere between an ideal crystal state and the amorphous state. The position of a crystal on a scale bounded by these 2 extremes is termed its crystallinity. A perfectly ordered crystal is an ideal state that is seldom, if

ever, achieved. The structural units of a crystal, termed unit cells, are repeated regularly and indefinitely in 3 dimensions in space. The unit cell has a definite orientation and shape defined by the translational vectors, a, b and c, and the angles α, β and γ, and hence has a definite volume, *V*, that contains the atoms and molecules necessary for forming the crystal. A crystalline system is defined by 3 long-range order symmetry operators (translational, orientational and conformational); the various mesophases (liquid crystals, crystals and plastic crystals) have 1 or 2 of the long-range Symmetry operators and the ideal amorphous state is defined by the absence of all 3 operators.

Each crystal can be classified as a member of one of 7 possible crystal systems or crystal classes that are defined by the relationships between the individual dimensions, a, b and c and between the individual angles, α, β and γ, of the unit cell. The structure of a given crystal may be classified according to one of the 7 crystal systems, to one of the 14 Bravais lattices and to one of the 230 space groups. All the 230 possible space groups, their symmetries and the symmetries of their diffraction patterns are compiled in the International Tables for Crystallography.

Many substances are capable of crystallizing in more than one type of crystal lattice, which is known as polymorphism. The occurrence of polymorphism is a common phenomenon among organic molecules, giving rise to different physicochemical properties. Crystalline polymorphs have the same chemical composition but different internal crystal structures and, therefore, possess different physico-chemical properties. The different crystal structures in polymorphs are due to different atomic packing arrangements and/or different conformations of the molecules.

The other extreme of a crystal state is the ideal or true amorphous state, where all long-range order is lost. For most organic systems certain short-range order remains, but this is not expected to extend over distances much larger than nearest neighbour (NN) or next nearest neighbour (NNN) interactions, which are typically less than 2-2.5 nm for small organic molecules.

Amorphous material is characterised by the absence of distinct reflections in the X-ray powder diffraction (XRPD) pattern.

The crystallinity of a real powder can be considered by 2 models of crystallinity. In the 1-state model all particles will be of the same crystallinity whereas in the 2-state model each particle can either be crystalline or amorphous, such that the actual crystallinity of the powder is the weighted average of these 2 extreme crystallinities. Such a powder is obtained when pure crystalline and amorphous phases are physically mixed. In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with different sizes and shapes.

The extent of disorder in a crystalline solid can affect many physico-chemical properties of substances for pharmaceutical use. Because of the great relevance of these properties, it is important to be able to assess the extent of disorder or the crystallinity of a solid by a suitable quantitative method.

Methods for Monitoring and Determining Crystallinity

Various methods are available for determining the crystallinity of a solid. Many techniques cannot detect or quantify these properties independently; for this reason, it is useful to combine several of the methods described below. Such methods often do not give accurate results and limits of quantitation are usually much greater than those for chemical impurities. In addition, certain assumptions have to be made about the relationship between standards used for calibration, which are typically mixtures of crystalline and amorphous particles (2-state model), and the samples to be analysed that are likely to have at least a small component of material exhibiting 1-state model behaviour. Finally, the lack of well-defined standards for 100 per cent crystalline or 100 per cent amorphous material complicates the validation of such methods. As explained above, it is obvious that different amorphous or non-crystalline phases exist and even co-exist in a solid powder. These different non-crystalline forms of a solid can give different responses depending on the techniques used for determining the degree of crystallinity.

X-ray powder diffraction. XRPD is still the most commonly used method for determining the degree of crystallinity, although this method suffers from some limitations due to peak broadening, amorphous halo and preferred orientation which make interpretation and quantitation difficult.

XRPD alone is often insufficient to distinguish between the different non-crystalline phases. The X-ray diffraction pattern of a purely amorphous and nanocrystalline phase is characteristic of a broad diffuse halo. In-depth analysis of the X-ray diffraction patterns will show that the diffuse halo in the pattern of nanocrystalline material shows some correlation to the pattern of the parent crystalline phase, while in the case of a pure amorphous phase such a correlation does not exist. Additional techniques may be required to establish the true nature of X-ray amorphous materials.

Thermal analysis. Thermal analysis of crystalline materials exhibits a melting transition that is often accompanied by decomposition or evaporation of solvents. In the case of true amorphous materials, thermal analysis reveals a glass transition, whereas only a melt would be expected for a nanocrystalline material.

Microcalorimetry. It is a highly sensitive technique which allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure. Amorphous parts of a substance can recrystallise by

subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The measurement of the heat of recrystallisation enables the amorphous content to be determined from the enthalpy of recrystallisation. By relating the output from the microcalorimeter for a sample to that obtained for an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1 per cent can be reached.

Solution calorimetry. Solution calorimetry provides a means of determining enthalpy of solution for a solid substance. The crystallinity of the solid sample to be examined is given by the enthalpy of solution of the solid sample (ΔH^s_x), minus the enthalpy of solution of the chosen reference standard of the same substance (ΔH^s_r), when determined under the same conditions. Because the reference standard is usually chosen for its perceived high crystallinity, its enthalpy of solution is usually algebraically greater (more endothermic or less exothermic) than that of the solid sample to be examined in the same solvent. Consequently, the crystallinity determined is a negative quantity with the SI units, kJ/mol or J/g (J/kg is avoided because of its unwieldiness and potential for error). The preference for a negative value with respect to a highly crystalline reference standard recognises the fact that most samples have a lower crystallinity than this reference standard.

Near-infrared (NIR) spectrophotometry. Near-infrared (NIR) spectrophotometry is another technique used to measure the degree of crystallinity, and has also been proven to be useful in studies of polymorphism. The NIR spectrum of a sample contains both physical and chemical information. Being non-invasive, non-destructive and operable at room temperature, the method is a valuable tool to assess changes in the amorphous and crystalline state.

Infrared absorption spectrophotometry and Raman spectrometry. Infrared absorption spectrophotometry (2.2.24) and Raman spectrometry are other techniques used to measure the degree of crystallinity, and have also been proven to be useful in studies of polymorphism. The IR spectrum and Raman spectrum of a sample contain both physical and chemical information.

Solid-state NMR. Solid-state nuclear magnetic resonance spectrometry (ss NMR) can be used to provide information about polymorphism and related relative molecular conformations. However, some caution has to be exercised in the interpretation of results, since it is not always simple to distinguish between samples that comprise a mixture of different physical forms (2-state model) and those that comprise crystals having disorder with exchange that is slow on the NMR timescale. Similarly, samples that contain defects arising from different molecular conformations or slightly different packing arrangements (1-state model) may show additional signals in the spectra. Solid-state NMR may be quite sensitive to this, even if lattice parameters are hardly affected and, consequently, little or no change is observed by XRPD. It is evident that the crystallinity of substances for pharmaceutical use can be complex, and both crystalline defects and amorphous material may co-exist.

Optical microscopy. A method to detect whether or not particles are crystalline is to use a polarising microscope, where particles show birefringence and extinction positions when the microscope stage is revolved.

2.4.38. Specific Surface Area

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

Brunauer, Emmett and Teller (BET) Theory and Specific Surface Area Determination

Multipoint Measurement

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$\frac{1}{\left[V_a\left(\frac{P_o}{P}-1\right)\right]}=\frac{C-1}{V_m C}\times\frac{P}{P_o}\times\frac{1}{V_m C} \qquad \text{---(1)}$$

Where, P = partial vapor pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in Pascal,

P_o = saturated pressure of adsorbate gas, in Pascal.

V_a = volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure (1.013×10^5 Pascal)], in ml,

V_m = volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in ml,

C = dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of V_a is measured at each of not less than three values of P/P_o.

Then the BET value

$$\frac{1}{\left[V_a\left(\frac{P_o}{P}-1\right)\right]}$$

is plotted against P/P_o, according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, r, of the linear regression is not less than 0.9975; that is, r^2 is not less than 0.995. From the resulting linear plot, the slope, which is equal to $(C-1)/V_mC$, and the intercept, which is equal to $1/V_mC$, are evaluated by linear regression analysis. From these values, V_m is calculated as $1/(slope + intercept)$, while C is calculated as $(slope/intercept)+1$. From the value of V_m so determined, the specific surface area, S, in $m^2 \cdot g^{-1}$, is calculated by the equation:

$$S = \frac{V_m N a}{m \times 22400} \qquad \text{---- (2)}$$

Where, N = Avogadro constant (6.022×10^{23} mol^{-1}),

a = effective cross-sectional area of one adsorbate molecule, in square meters (0.162 nm^2 for nitrogen and 0.195 nm^2 for krypton),

m = mass of test powder, in g,

22,400 = volume, in ml, occupied by one mole of the adsorbate gas at STP allowing for minor departures from the ideal.

A minimum of three data points is required. Additional measurements may be carried out especially when nonlinearity is obtained at a P/P_o value close to 0.3. Because nonlinearity is often obtained at a P/P_o value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

Single-Point Measurement

Normally, at least three measurements of V_a, each at different values of P/P_o, are required for the determination of specific surface area by the dynamic flow gas adsorption technique *(Method I)* or by volumetric gas adsorption *(Method II)*. However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of V_a measured at a single value of P/P_o such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating V_m:

$$V_m = V_a\left(1-\frac{P}{P_o}\right) \qquad \text{---- (3)}$$

The specific surface area is then calculated from the value of V_m by equation (2) given above.

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant C is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multipoint method for the series of powder samples. Close similarity between the single-point values and multipoint values suggests that 1/C approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant C is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multipoint method to evaluate C for one of the samples of the series from the BET plot, from which C is calculated as $(1 + slope/intercept)$. Then V_m is calculated from the single value of V_a measured at a single value of P/P_o by the equation:

$$V_m = V_a\left(\frac{P_o}{P}-1\right)\left[\frac{1}{C}+\frac{C-1}{C}\times\left(\frac{P}{P_o}\right)\right] \qquad \text{---- (4)}$$

The specific surface area is calculated from V_m by equation (2) given above.

Experimental Techniques

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique *(Method I)* and the volumetric gas adsorption technique *(Method II)*.

Sample Preparation

Outgassing

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapors that may have become physically adsorbed onto the surface after manufacture and during treatment, handling, and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapors. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

Condition

The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure, and time are chosen so that the original surface of the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum by purging the sample in a flowing stream of a nonreactive, dry gas or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

Adsorbate

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area (< 0.2 m^2g^{-1}), the proportion adsorbed is low. In such cases, the use of krypton at the liquid nitrogen temperature is preferred because the low vapor pressure exerted by this gas greatly reduces error. The use of larger sample quantities, where feasible (equivalent to 1 m^2 or greater total surface area using nitrogen), may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

Quantity of Sample

A quantity of the test powder is accurately weighed such that the total surface of the sample is at least 1 m^2 when the adsorbate is nitrogen and 0.5 m^2 when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

Measurements

Because the amount of gas adsorbed under a given pressure tends to increase when the temperature is decreased, adsorption measurements are usually made at a low temperature. Measurement is performed at 77.4 K, the boiling point of liquid nitrogen.

Method I. The Dynamic Flow Method

Principle

In the dynamic flow method (see Fig. 2.4.38-1), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

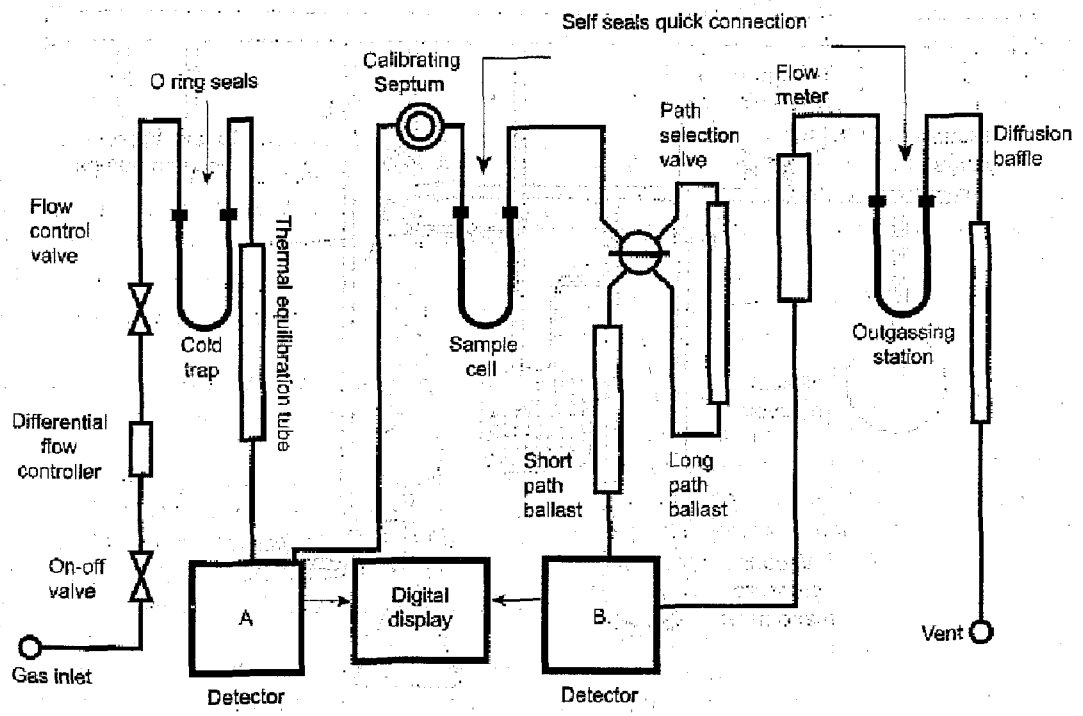


Fig. 2.4.38-1: Schematic diagram of the dynamic flow method apparatus

A minimum of three mixtures of the appropriate adsorbate gas with helium are required within the P/P_o range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of three data points within the recommended range of 0.05 to 0.30 for P/P_o is determined.

Procedure

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample again, through the thermal conductivity cell, and then to a recording potentiometer.

The sample cell is immersed in liquid nitrogen, and the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

The sample is removed from the coolant; this gives desorption peak equal in area and in the opposite direction to the adsorption peak. Because this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, a known quantity of adsorbate, sufficient to give a peak of similar magnitude to the desorption peak, is injected into the system, and the proportion of gas volume per unit peak area is obtained.

A mixture of nitrogen and helium is used for a single-point determination; and several such mixtures or premixing two streams of gas are used for a multipoint determination.

The calculation is the same as the volumetric method.

Method II. The Volumetric Method

Principle

In the volumetric method (see Fig. 2.4.38-2), the recommended adsorbate gas is nitrogen, which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P, of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Because only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

Procedure

A small amount of dry nitrogen is admitted into the sample tube to prevent contamination of the clean surface, the sample tube is removed, a stopper is inserted, the tube is weighed, and the weight of the sample is calculated. Then the sample

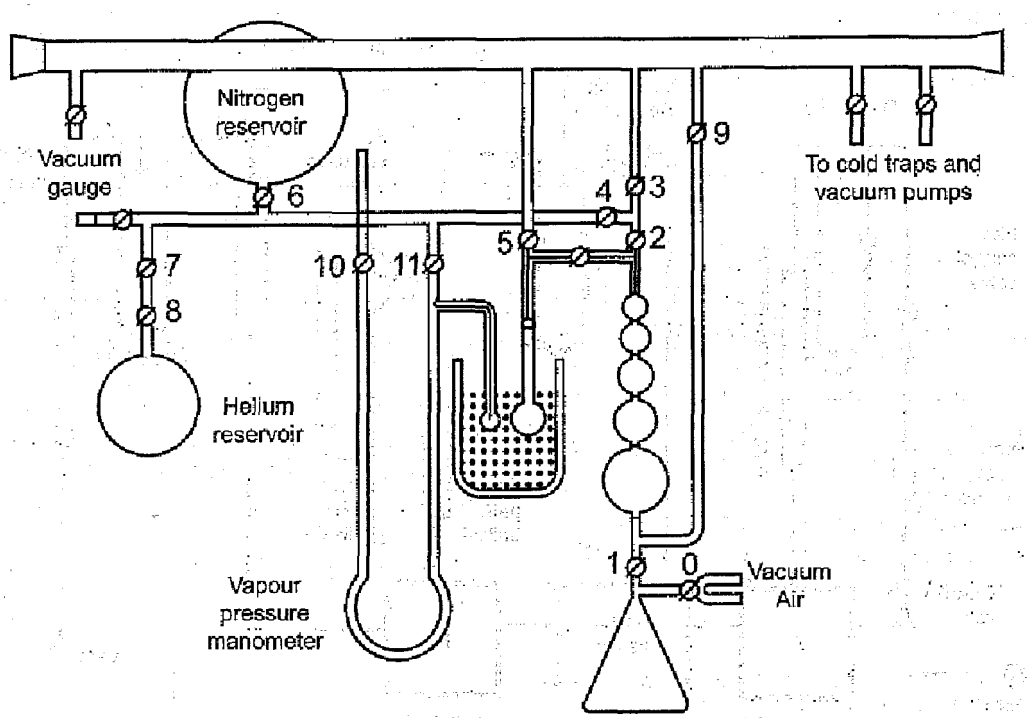


Fig. 2.4.38-2: Schematic diagram of the volumetric method apparatus

tube is attached to the volumetric apparatus. The sample is cautiously evacuated down to the specified pressure (e.g., between 2 Pa and 10 Pa). Alternately, some instruments are operated by evacuating to a defined rate of pressure change (e.g., less than 13 Pa/30 s) and by holding for a defined period of time before commencing the next step.

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a non-adsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements: that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed, V_a. For multipoint measurements, repeat the measurement of V_a at successively higher P/P_o values. When nitrogen is used as the adsorbate gas, P/P_o values of 0.10, 0.20, and 0.30 are often suitable.

Reference Materials

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area such as α-alumina, which should have a specific surface area similar to that of the sample to be examined.

2.4.39. Mass Spectrometry

A mass spectrometer produces ions from the substance under investigation, separates them according to their mass-to-charge ratio (m/z), and records the relative abundance of each ionic species present. The instrument consists of three major components (see Fig. 2.4.39-1).

- an ion source for producing gaseous ions from the substance being studied,
- an analyzer for resolving the ions into their characteristic mass components according to their mass-to-charge ratios,and
- a detector system for detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10^{-6} to 10^{-8} mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

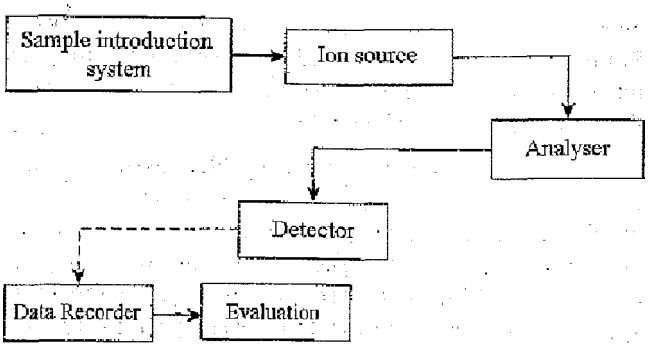


Fig. 2.4.39-1: Major components of a mass spectrometer

This chapter gives an overview of the theory, construction, and use of mass spectrometers. The discussion is limited to those instruments and measurements with actual or potential application to compendial and other pharmaceutical requirements: generally, the identification and quantitation of specific compounds.

Sample Introduction

Samples are introduced either as a gas to be ionized in the ion source, or by ejection of charged molecular species from a solid surface or solution. In some cases sample introduction and ionization take place in a single process, making a distinction between them somewhat artificial.

Substances that are gases or liquids at room temperature and atmospheric pressure can be admitted to the source as a neutral beam via a controllable leak system. Volatilizable compounds dissolved or adsorbed in solids or liquids can be removed and concentrated with a headspace analyzer. Vapour are flushed from the solid or liquid matrix with a stream of carrier gas and trapped on an adsorbing column. The trapped vapour are subsequently desorbed by programmed heating of the trap and introduced into the mass spectrometer by a capillary connection.

For volatilizable solids, the most frequently used method of sample introduction is the direct insertion probe. Here, the sample is placed in a small crucible at the tip of the probe, which is heated under high vacuum in close proximity to the ion source. A variation of this technique involves desorption of samples inside the ionization chamber from a rapidly heated wire or with the aid of a laser beam. Such desorption techniques, in combination with electron, chemical, or field ionization, are preferred for the analysis of heat sensitive or poorly volatile samples.

Sample introduction techniques that involve the ejection of charged molecules from the surface of solid samples include the field desorption method and various sputtering techniques, where the samples are bombarded by high energy

photons, by a primary ion beam, or by a neutral particle beam. Similarly, ions can be ejected from solutions either by bombardment with a primary beam, or by one of the various spray techniques described below.

Gas and liquid chromatographs are widely used as sample inlet devices for mass spectrometers. These chromatographs provide for an initial sample purification, since only that portion of the chromatographic effluent containing the compound of interest need be admitted to the mass spectrometer. Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) combinations are valuable tools for the identification of unknown impurities in drugs. These combination methods have the capacity to separate complex mixtures with the opportunity to obtain structural information on the individual components.

Gas Chromatography/Mass Spectrometry

Gas chromatographic effluents are already in the vapor state and can be admitted directly into the mass spectrometer. Bridging the several orders of magnitude difference in the operating pressures of the two systems was initially accomplished with the use of various carrier gas separators. However, with the advent of capillary gas chromatographic columns and high capacity vacuum pumps for mass spectrometers, the gas chromatographic effluents are now fed directly into the ion source.

Liquid Chromatography/Mass Spectrometry

This technique is particularly useful for analyzing materials that cannot be analyzed by GC/MS, either because of thermal instability, high polarity, or high molecular weight. Compounds of biological interest such as drugs and their metabolites, polar endogenous substances, and macromolecules-including peptides, proteins, nucleic acids, and oligosaccharides-often fall into one of these categories.

Currently available LC/MS interfaces encompass a number of approaches to separating the compound of interest from the liquid chromatographic mobile phase and transforming it into an ionized species suitable for mass spectrometry. These include transport devices such as the particle beam; various spray techniques including thermospray, electrospray, and ionspray; and particle-induced desorption such as continuous-flow fast atom bombardment (CF-FAB).

Particle Beam Interface

The solvent is removed from an aerosol of the liquid chromatographic effluent, and the resulting neutral analyte molecules are introduced into the ion source of the mass spectrometer where they are ionized by electron ionization (EI) or chemical ionization (CI). The resulting spectra are thus classical EI or CI spectra, the former with a wealth of structural information. There are limitations with respect to polarity, thermal lability, and molecular weight, so this technique is best suited for small organic molecules with molecular weights of less than 1000 daltons.

Thermospray

The compound of interest in a volatile buffer mobile phase, such as ammonium acetate, is passed through heated, narrow bore tubing directly into the ion source of a mass spectrometer. The solution is vaporized in the tubing, and analyte ions desorb into the gas phase and pass into the mass analyzer. Neutral analyte molecules in the gas phase may undergo chemical ionization by reaction with gas phase buffer ions such as NH_4^+. Thermospray is compatible with relatively high flow rates of 1 to 2 ml per minute, solvents containing a high percentage of water, and many types of polar analytes. Thermal degradation may occur, since the analytes are exposed to relatively high temperatures during the volatilization process.

Electrospray

The mobile phase is sprayed through a small opening (needle tip) held at a potential of several kilovolts. The charged droplets so produced are desolvated by passing through a drying gas, and the resulting ions are injected directly into the high vacuum of the analyzer through an orifice or glass capillary. Classical electrospray is limited to flow rates of 1 to 5 µl per minute, and is therefore compatible with either microbore HPLC or post-column stream splitting techniques.

The ions may carry multiple charges, so that the m/z value of high molecular weight substances will fall into the usable range for most quadrupole or magnetic sector mass analyzers ($m/z < 4000$). Analytes of up to 150,000 daltons can thus be successfully analyzed.

Ionspray

A variant of electrospray in which nebulization with a gas flow is used to assist the formation of microdroplets of mobile phase. The technique can extend the upper limit of usable flow rates to 0.1 ml per minute. Volatile buffers must be used with both techniques.

Desorption Techniques

Microflow liquid chromatography can also be interfaced to particle induced desorption techniques such as fast atom bombardment (FAB) and liquid secondary ion mass spectroscopy (LSIMS), described in the following section on ionization techniques. Typically, column effluent flowing at a rate of 1 to 10 µl per minute is mixed with a small percentage of nonvolatile liquid such as glycerol. The mixture is introduced via a capillary inlet onto a target within the ion source where it is bombarded with high energy (5 to 20 keV) atoms or ions.

The resulting spectra are similar to FAB or LSIMS spectra but with the background from the sample matrix greatly reduced. Frit-FAB is a variant of continuous flow FAB where the sample is introduced through a porous frit target.

Ionization Techniques

Electron Impact

Molecules of the sample under analysis enter the ionization chamber in the vapor state. Positive ions are produced by passing a beam of electrons, obtained from tungsten or rhenium filaments, through the vapor, which is maintained at a pressure of 10^{-4} to 10^{-6} mm of mercury. Provided the energy of the electron beam is greater than the ionization potential of the sample, the sample is ionized and/or fragmented, as represented by the following equation: $e^- + M \rightarrow M^+ + 2e^-$.

Chemical Ionization (CI)

In this process, a reagent gas at a pressure between 0.1 to 10 mm of mercury is admitted to the source and ionized by a high energy electron beam or discharge. At these pressures, ion-molecule reactions occur and the primary reagent gas ions react further. The most commonly used reagent gases are methane, isobutane, and ammonia. Typical reactions for methane are shown in the following equations:

$$CH_4 + e^- \rightarrow CH_4^+ + 2e^-$$

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$$

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$

The CH_5^+ species is a strong Bronsted acid and readily transfers a proton to most organic compounds

$$CH_5^+ + M \rightarrow MH^+ + CH_4$$

In the case of methane, the protonated ion $(MH)^+$ initially formed may be sufficiently energetic to dissociate further.

Fast Atom Bombardment (FAB)

The sample is ionized by bombardment with a beam of high speed xenon atoms, produced by exchange with highly accelerated xenon ions in a collision cell. The process is summarized as follows:

$$Xe \rightarrow Xe^+ + e^-$$

$$\underrightarrow{Xe}^+ + Xe \rightarrow \underrightarrow{Xe} + Xe^+ ,$$

where the subscript arrows indicate the fast-moving particles.

FAB is a surface analysis technique, and care must be taken during sample preparation to optimize the condition of the surface. When the sample is coated on a probe by evaporation of a solution, the sample ion beam obtained is often transitory. Molecular adducts with alkali metals, such as (M + Na) and (M + K), favor ion formation. This phenomenon is used to assist in the ionization of biological molecules. Thus, treatment of the sample surface with sodium chloride solution may enhance the yield of adduct ions. Heating the sample during analysis may also increase the ion yield.

The declining yield of sample ions during analysis is probably due to destruction of the sample surface. The surface can, in effect, be continuously replaced by dissolving the sample in a suitable nonvolatile liquid and by coating the mixture onto the top of the probe. Using this approach, the lifetime of samples in the source has been extended to more than 1 hour and the range of compounds amenable to FAB analysis expanded dramatically. The long sample lifetimes and higher sensitivities so achieved make FAB an important mass spectral technique for biochemical analysis, providing the elemental formula of the sample through accurate mass determination. A further advantage of FAB, unlike CI, is the presence of fragment ions within the spectra, which aid in structural elucidation.

Recently, neutral atom bombardment has been replaced by cesium ion bombardment. Although this technique is still referred to as FAB, it is more correctly described as liquid secondary ion mass spectrometry (LSIMS).

Negative and positive ions are formed in the various ionization processes described above, and both are readily analyzed by modern mass spectrometers. Samples with a high electron capture cross section, notably those containing halide atoms, yield an abundance of negative ions. For this reason, halide derivatives of compounds to be studied are often prepared. Negative ion mass spectrometry has been successfully applied to the analysis of pesticide residues, since the structures of these compounds are well suited to the technique.

Analyzers

Mass analyzers separate the charged species in the ionized sample according to their m/z ratios, thus permitting the mass and abundance of each species to be determined. Four commonly used analyzers are the magnetic sector, the quadrupole, the time-of-flight, and the Fourier transform analyzers.

Magnetic Sector Analyzers

Ions generated in the ion source are collimated into a beam through the focusing action of a magnetic field and a slit assembly. After exiting the source, ions are subjected to a magnetic field perpendicular to the direction of the beam. Each ion experiences a force at right angles to both its direction of travel and the direction of the magnetic field, thereby deflecting the beam. The motion of each ion is given by

$$m/z = H^2r^2/2V$$

where m is the mass in atomic mass units, z is the number of electronic charges, H is the magnetic field strength in gauss, r

is the radius of the ion trajectory in centimeters, and V is the accelerating voltage. The mass spectrum is scanned by varying the strength of the magnetic field and detecting those ions passing through an exit slit as they come into "focus." The magnetic sector mass spectrometer affords spatial resolution of ions, giving a unique trajectory at a given field strength for each value of m/z.

Quadrupole Analyzers

The instrument is based on four parallel rods in a square array. The ion beam is focused down the axis of the array and an electrical potential of fixed (DC) and radio frequency (RF) components is applied to diagonally opposed rods. For a given combination of DC and RF components, ions of one specific m/z ratio have a stable path down the axis. All others are deflected to the sides and lost. Mass scanning is achieved by changing the DC and RF components of the voltage, while maintaining a fixed ratio. The quadrupole analyzer is a mass filter because it separates ions on the basis of their m/z ratio.

Ion-Trap Analyzer

This quadrupole-type device is composed of a ring electrode placed between two end cap electrodes. Depending upon the commercial version employed, the end caps are either held at ground potential or have an RF voltage applied to them, while an RF voltage is placed on the ring electrode. As a result of these potentials, the hyperbolic surfaces of the three elements form a three-dimensional quadrupole analyzer.

Both ionization and mass analysis take place within the three-dimensional quadrupole field. In the ionization step, the RF voltage on the ring electrode is set low enough so that the ions within the mass range of interest are trapped within the device. Following ionization, mass analysis is accomplished through use of the "mass selective instability" mode of operation. That is, by raising the RF voltage on the ring electrode, ions of successively higher mass are ejected from the ion trap into an electron multiplier detector. In its most common application, the ion-trap analyzer is used in conjunction with a gas chromatograph and covers the mass range of 10 to 560 daltons. However, recent advances in ion-trap technology have extended the workable mass range to many thousands of daltons.

Time-of-Flight Analyzers

Ion separation is based on the principle that ions of different masses, possessing equal kinetic energy, have different velocities. If there is a fixed distance for the ions to travel, the time of travel will vary with their mass, the lighter ions traveling faster and reaching the detector in a shorter period of time. The time-of-flight is given by

$$t_f = k\sqrt{m/z}$$

where t_f is the time-of-flight in seconds. Thus, the time-of-flight of the various ions is simply proportional to the square root of the mass-to-charge ratio of the ions. To measure the time-of-flight, ions are introduced into the mass spectrometer in discrete packets so that a starting point for the timing process can be established. Ion packets are generated either through a pulsed ionization process or through a gating system in which ions are produced continuously, but are introduced only at given times into the flight tube.

Fourier Transform Analyzers

In a magnetic field of flux density B, ions move in circular orbits. The angular frequency, ω, of the orbital motion is given by

$$\omega = (z/m)B$$

In this type of mass spectrometer, the orbits are varied by subjecting the ions to a resonant alternating electric field. When the frequency of the alternating field matches the orbital frequency, the ions are steadily accelerated to larger and larger orbits in coherent motion, developing a high level of kinetic energy. After the alternating electric field is turned off, the orbiting ions give rise to an alternating image current on the electrodes. A frequency analysis of this signal yields the mass of the ions involved. Thus, the Fourier transform of the time domain transient signal yields the corresponding frequency spectrum from which the mass spectrum is computed. This is a high resolution technique, yielding m/z ratios accurate to about one thousandth of a dalton.

Tandem Mass Spectrometry

Two mass spectrometers connected in series (MS/MS), tandem mass spectrometry, refers to the use of two or more sequential mass analysis steps. In its simplest form MS/MS (Fig. 2.4.39-2)

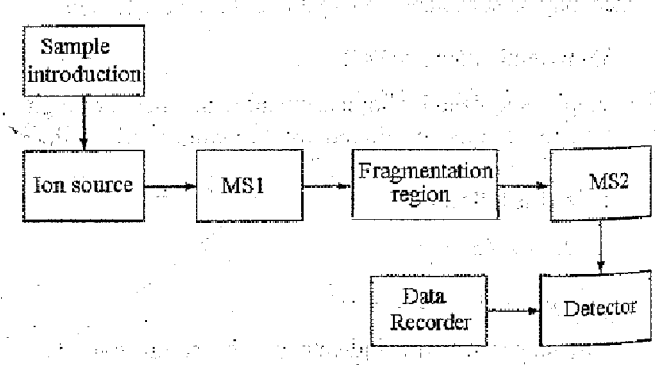


Fig. 2.4.39-2: Tandem mass spectrometry

consists of two mass spectrometers linked in such a way that ions preselected by the first mass analyzer (MS1) are chemically or energetically modified and the results analyzed by the second mass analyzer (MS2).

The basic concept of MS/MS involves the ability to determine the mass relationship between a precursor ion in MS1 and a product ion in MS2. Different mass relationships can be probed depending on how MS1 and MS2 are scanned. These include fragmentation of a precursor and measurement of all its fragments (a product scan), selection of multiple precursors and testing for a common fragment (a precursor scan), or scanning to see if a number of precursors all lose the same neutral species (a constant neutral loss scan).

Fragmentation of the precursor ion can be induced by momentum transfer through collision with gas molecules and/or solid surfaces or by electronic excitation using lasers. These techniques are known as collision-induced dissociation, surface-induced dissociation, or laser-induced dissociation, respectively. Allowing the ion to fragment without additional activation is known as metastable decomposition.

There are many applications of MS/MS to pharmaceutical problems. Product scans can be used to obtain qualitative information from precursor ions of drug substances, impurities, and contaminants: This can aid in the identification of unknowns. The method can also be used to determine the amino acid sequence of peptides and protein fragments.

MS/MS has advantages for mixture analysis. Even when the mass spectrometer is coupled to a separation device such as a liquid or gas chromatograph, the resulting signals may be a result of overlapping or unresolved components. MS/MS can be employed to select the precursor ion from one component and obtain structural information without interference from the others.

Selected reaction monitoring is used to reduce the interference encountered during quantitative analysis for low levels of drugs in biological matrices, as in pharmacokinetic studies. If analysis is for a drug specific ion, interfering signals from other compounds in the matrix can mask the desired signal. Interference is reduced if a drug-specific fragment is selected with MS1 and a structure-specific fragment with MS2. The odds of another molecule producing the same mass relationship are diminishingly small.

MS/MS can also be used in metabolism studies to search for molecules with common structural features such as metabolites related to the drug substance. All of the metabolites might contain the same functional group that is lost as a neutral fragment. In this case a contant-neutral-loss scan will show all of these species. For instance, carboxylic acids will all lose neutral carbon dioxide. If the common functionality is lost as an ionic fragment, then a precursor scan will show all of the molecules that produce that fragment ion.

Data Analysis and Interpretation

The mass spectral experiment yields information on the molecular weight of ions derived from the sample and the relative abundance of each of these ions. Spectra are often complex, and not all of the ions may be separated by the mass spectrometer. The ability of the instrument to separate ions is called the resolving power, commonly described by the "10 per cent valley" definition. This states that the resolving power is the highest mass number at which two peaks differing by one molecular weight unit and of equal height have a valley between them that is equal to 10 per cent of the peak height. For low, medium, and high resolution mass spectrometers, this value is between 100 and 2,000, 2,000 and 10,000, and greater than 10,000, respectively.

If one electron is removed or added to a neutral molecule, a molecular ion of essentially the same molecular weight as the parent molecule results. It is often possible to determine the mass of this ion with sufficient precision to enable the empirical formula of the compound to be calculated. Molecular masses may be determined accurately by using high resolution instruments or by peak-matching measurements using reference compounds.

Fragment ions are those produced from the molecular ion by various bond cleavage processes. Numerous papers in the literature relate bond cleavage patterns (fragmentation patterns) to molecular structure.

In addition to measurement of the mass of a molecular ion and its associated fragment ions, mass spectrometers are also used to quantitate compounds with a high degree of selectivity, precision, and accuracy. Compounds are introduced into the mass spectrometer either via direct insertion probe, gas inlet, or, as is more common, via gas or liquid chromatographic interfaces, which provide sample purification. Ionization may be by EI, CI, FAB, thermospray, or electrospray and mass separation by magnetic sector, quadrupole, or quadrupole ion-trap mass spectrometers. Quantitative mass spectrometry involves measuring the abundance of a specific ion, or set of ions, and relating the response to a known standard. External or internal standards may be used, but the latter are preferred for greater accuracy.

For mass spectrometry, internal standards may be either structural or stable isotope analogs. The former have the advantage of lower cost and availability while precision and accuracy are typically achieved by use of a stable isotope (^{2}H, ^{13}C, ^{15}N) labeled analog of the analyte. The only requirements for labeling the analyte are that the ion monitored for the internal standard must retain an isotopic label after ionization and the label must not be exchangeable under the sampling, separation, or ionization conditions. Stable isotope internal standards are often required for acceptable quantitation, particularly with FAB and LC/MS techniques such as thermospray and electrospray.

Relative abundances of the analyte and internal standard ions are typically determined by selected ion monitoring, by which

only specific ions due to the analyte and the internal standard are monitored. This technique has the advantage over scanning the full mass range in that more time is spent integrating the ion current at a selected mass-to-charge ratio, thereby increasing sensitivity. Chromatographic peak area or amount of analyte in a sample is calculated from the ratio of analyte to internal standard peak area (or height) and the regression parameters as determined by a calibration curve, using standard techniques.

2.4.40 Ethylene Oxide and Dioxan

The test is intended for the determination of residual *ethylene oxide* and *dioxan* in samples soluble in *water* or *dimethylacetamide*. For substances that are insoluble or insufficiently soluble in these solvents, the preparation of the sample solution and the head-space conditions to be employed is given in the individual monograph. Examine by head-space gas chromatography (2.4.13).

Method A

NOTE —This method is applicable for samples soluble in or miscible with water.

Test solution. Weigh 1.0 g of the substance under examination in a vial and add 1.0 ml of *water*. Mix and allow to stand at 70° for 45 minutes.

Reference solution (a). Weigh accurately 1.0 g of the substance under examination into a vial, add 0.5 ml of *ethylene oxide solution 3* and 0.5 ml of *dioxan solution1*. Mix and allow to stand at 70° for 45 minutes.

Reference solution (b). To 0.5 ml of *ethylene oxide solution 3* in a vial add 0.1 ml of a freshly prepared 10 mg per litre solution of *acetaldehyde* and 0.1 ml of *dioxan solution* 1. Mix and allow to stand at 70° for 45 minutes.

Method B

NOTE —This method is applicable for samples soluble in or miscible with dimethylacetamide.

Test solution. Weigh 1.0 g of the substance under examination in a vial and add 1.0 ml of *dimethylacetamide* and 0.20 ml of *water*. Mix and allow to stand at 90° for 45 minutes.

Reference solution (a). Weigh 1.0 g of the substance under examination into a vial, add 1.0 ml of *dimethylacetamide*, 0.1 ml of *dioxan solution* and 0.1 ml *of ethylene oxide solution 2*. Mix and allow to stand at 90° for 45 minutes.

Reference solution (b). To 0.10 ml of *ethylene oxide solution 2* in a vial, add 0.1 ml of a freshly prepared 10 mg/l solution of *acetaldehyde* and 0.10 ml of *dioxan solution*. Mix and allow to stand at 70° for 45 minutes.

The following static head-space injection conditions may be used:

- equilibration temperature: 70° (90° for solutions in *dimethylacetamide*),
- equilibration time: 45 minutes,
- transfer-line temperature: 75° (150° for solutions in *dimethylacetamide*),
- carrier gas: *helium* for chromatography,
- pressurisation time: 1 minutes,
- injection time: 12 seconds.

Chromatographic system

- a capillary glass or quartz column 30 m x 0.32 mm, coated with a 1.0 µm thick layer of *poly(dimethyl)siloxane*,
- Carrier gas: *helium* or *nitrogen* for chromatography,
- linear velocity: about 20 cm/seconds,
- split ratio: 1:20,
- flame-ionisation detector,
- temperature: column at 50° for 5 minutes, then raised to180° at a rate of 5° per minute, again raised to 230° at a rate of 30° per minute and maintaining at 230° for 5 minutes.
- inlet port at 150° and detector at 250°,

Inject a suitable volume, for example 1.0 ml, of the gaseous phase of reference solution (b) into the column adjust the sensitivity of the system so that the heights of the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained are at least 15 per cent of the full scale of the recorder. Inject separately suitable volumes, for example 1.0 ml (or the same volume used for reference solution (b), of the gaseous phases of the test solution and reference solution (a). Repeat the procedure twice more.

System suitability

The test is not valid unless the resolution between the peaks corresponding to acetaldehyde and ethylene oxide is at least 2.0 and the peak of dioxan is detected with a signal-to-noise ratio of at least 5. For each pair of injections, calculate for ethylene oxide and for dioxan the difference in area between the peaks obtained with the test solution and reference solution (a). The test is not valid unless the relative standard deviation of the 3 values obtained for ethylene oxide is not more than 15 per cent and the relative standard deviation of the 3 values obtained for dioxan is not more than 10 per cent. If the weighings used for the test solution and reference solution differ from 1.0 g by more than 0.5 per cent, the appropriate corrections must be made.

The content of ethylene oxide or dioxan in parts per million is calculated from the expressions:

$$\frac{AT \times C}{(AR \times MT) - (AT \times MR)}$$

where, AT = area of the peak corresponding to ethylene oxide in the chromatogram obtained with the test solution,

AR = area of the peak corresponding to ethylene oxide in the chromatogram obtained with reference solution (a),

MT = mass of the substance under examination in the test solution, in grams,

MR = mass of the substance under examination in the reference solution, in grams,

C = the amount of ethylene oxide added to reference solution (a), in micrograms.

$$\frac{DT \times C}{(DR \times MT) - (DT \times MR)}$$

where, DT = area of the peak corresponding to dioxan in the chromatogram obtained with the test solution,

DR = area of the peak corresponding to dioxan in the chromatogram obtained with reference solution (a),

C = the amount of dioxan added to reference solution (a) in micrograms.

2.4.41. Acetic Acid in Peptides

NOTE —The following procedure is to be used to determine the amount of acetate or acetic acid in peptides. Acetate is a common counter ion in many peptide preparations.

Strong Sodium Hydroxide Solution. Dissolve 42 g of *sodium hydroxide* in *water*, and dilute with *water* to 100.0 ml.

Solvent mixture. 95 volumes of mobile phase A and 5 volumes of *methanol*.

Reference solution. A 0.01 per cent v/v solution of *glacial acetic acid RS* in *solvent mixture*.

(The concentration can be adjusted depending on the amount of acetate or acetic acid expected to be present in the test material.)

Test solution. Prepare as directed in the individual monograph. The amount of material used can be adapted depending on the amount of *acetic acid* expected.

Chromatographic system

- a stainless steel column 25cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. add 0.7 ml of *orthophosphoric acid* to 1000 ml of *water*, and adjust with *strong sodium hydroxide* solution to a pH of 3.0,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrometer set at 210 nm,
- Injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
10	50	50
20	50	50
22	95	5

Inject reference solution and the test solution. The test is not valid unless the retention time of acetic acid is not between 3 and 4 minutes, and the relative standard deviation for replicate injections is not more than 5 per cent.

Calculate the content of acetic acid.

2.4.42. Inductively Coupled Plasma Spectrometry

Inductively Coupled Plasma Spectrometry is a plasma-based instrumental technique, which is useful for pharmaceutical analyses. The inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma–atomic emission spectroscopy (ICP-AES; also referred to as inductively coupled plasma optical emission spectroscopy (ICP-OES)), or the excited or ground state ions can be determined by a technique known as inductively coupled plasma–mass spectrometry (ICP-MS). ICP-AES and ICP-MS may be used for either single- or multi-element analysis and used for either sequential or simultaneous analyses with good sensitivity over an extended linear range.

Inductively Coupled Plasma-Mass Spectrometry

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) is a mass spectrometry method that uses an inductively coupled plasma (ICP) as the high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. ICP-MS utilizes the ability of the ICP to generate charged ions from the element species within a sample. These ions are then directed into a mass spectrometer, which separates them according to their mass-to-charge ratio (m/z). Most mass spectrometers have a quadrupole system or a magnetic sector. Ions are transported from the plasma through 2 cones (sampler and skimmer cones, forming the interface region) to the ion optics. The ion optics

consist of an electrostatic lens, which takes ions from an area at atmospheric pressure to the mass filter at a vacuum of 10-8 Pa or less, maintained with a turbo molecular pump. After their filtration, ions of the selected mass/charge ratio are directed to a detector (channel electromultiplier, Faraday cup, dynodes), where ion currents are converted into electrical signals. The element is quantified according to the number of ions arriving and generating electrical pulses per unit time.

Apparatus

Inductively coupled plasma-mass spectrometer (ICP-MS) consists of sample-introduction system with peristaltic pump for delivering the solution at constant flow rate into a nebuliser, radio-frequency (RF) generator, plasma torch. interface region including cones to transport ions to the ion optics, mass spectrometer, detector and a data-acquisition unit.

Interference

The prime characteristic of an ICP-MS instrument is its resolution, i.e. the efficiency of separation of two close masses. Quadrupole instruments are, from this point of view, inferior to magnetic-sector spectrometers. Mass interference is the major problem, for example by isobaric species that significantly overlap the mass signal of the ions of interest, especially in the central part of the mass range (for example 40-80 a.m.u.). The combination of atomic ions leads to polyatomic or molecular interferences (i.e. ^{40}Ar, ^{16}O with ^{56}Fe or ^{40}Ar, ^{40}Ar with ^{80}Se). Matrix interference may also occur with some analytes. Some samples have an impact on droplet formation or on the ionization temperature in the plasma. These phenomena may lead to the suppression of analyte signals. Physical interference is to be circumvented by using the method of internal standardization or by standard addition. The element used as internal standard depends on the element to be measured: ^{59}Co and ^{115}In, for example, can be used as internal standards.

Procedure

Sample Preparation

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP-MS. The most conventional means by which samples are introduced into the plasma is via solution nebulization. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulphuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. The sample preparation usually involves a step of digestion of the matrix by a suitable method, for example in a microwave oven. Furthermore, it is important to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Sample Introduction

There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used. Several sample-introduction systems tolerate high acid concentrations, but the use of sulphuric and phosphoric acids can contribute to background emission. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps usually deliver the standard and sample solutions at a rate of 20-1000 µl per minute.

Operating Conditions

Follow the procedure as directed in the individual monograph for the instrumental parameters. The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen as selection of cones (material of sampler and skimmer), support-gas flow rates (outer, intermediate and inner tubes of the torch), RF power, pump speed, selection of one or more isotopes of the element to be measured (mass). Optimization of an ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

Selection of Isotope

The selection of the analytical mass to use is critical to the success of an ICP-MS analysis. The most abundant isotope

for a given element is selected to obtain maximum sensitivity. Furthermore, an isotope with the least interference from other species in the sample matrix and from the support gas should be selected. Information about isobaric interferences and interferences from polyatomic ions of various types, for example hydrides, oxides, chlorides, etc., is usually available in the software of ICP-MS instrument manufacturers.

Control of Instrument Performance

System suitability

Tuning of the instrument allows to monitor and adjust the measurement before running samples. ICP-MS mass accuracy is checked with a tuning solution containing several isotopes covering the whole range of masses, for example ^{9}Be, ^{59}Co, ^{89}Y, ^{115}In, ^{140}Ce and ^{209}Bi. Sensitivity, short- and long-term stability are recorded. The instrument parameters (plasma condition, ion lenses and quadrupole parameter) are to be optimized to obtain the highest possible number of counts. Tuning for resolution and mass axis is to be done with a solution of Li, Y and Tl to ensure an acceptable response over a wide range of masses. Evaluation of the efficiency of the plasma to decompose oxides has to be performed in order to minimize these interferences. The ratio Ce/CeO and/or Ba/BaO is a good indicator, and a level less than about 3 per cent is required. Reduction of the formation of double-charged ions is made with Ba and Ce. The ratio of the signal for double-charged ions to the assigned element should be less than 2 per cent. Long-term stability is checked by running a standard first and at the end of the sample sequence, controlling whether salt deposits on the cones have reduced the signal throughout the run.

Method Validation

One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

Linearity

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates. The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is not valid unless the correlation coefficient is at least 0.99. the residuals of each calibration level are randomly distributed around the calibration curve. Calculate the mean and relative standard deviation for the lowest and for the highest calibration level. When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

Accuracy

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

Repeatability

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

Limit of Quantification

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

Limit of Detection

Verify that the limit of detection (for example, determined using the 3σ approach) is below the value to be measured.

Inductively Coupled Plasma-Atomic Emission Spectrometry

Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) is an atomic emission spectrometry method that uses an inductively coupled plasma (ICP) as the excitation source. An ICP is a highly ionised inert gas (usually argon) with equal numbers of electrons and ions sustained by a radio-frequency (RF) field. The high temperature reached in the plasma successively desolvates, vaporises, excites - atomic emission spectrometry (AES) detection - and ionises - mass spectrometry (MS) detection - atoms from the sample. Detection limits are, generally, in the lower nanogram (ICP-MS) to microgram (ICP-AES) per litre range.

The plasma is formed by a tangential stream of support gas through a 'torch', i.e. a system consisting of 3 concentric

quartz tubes. A metal coil (the load coil) surrounds the top end of the torch and is connected to a radio-frequency (RF) generator. Power (usually 700-1500 watt) is applied through the coil and an oscillating magnetic field corresponding to the frequency of the generator (in most cases 27 megahertz, 40 megahertz) is formed. The plasma forms when the support gas is made conductive by exposing it to an electric discharge, which produces seed electrons and ions. Inside the induced magnetic field, the charged particles (electrons and ions) are forced to flow in a closed annular path. As they meet resistance to their flow, heating takes place producing additional ionisation. The process occurs almost instantaneously, and the plasma expands to its full strength and dimensions. The radio-frequency oscillation of the power applied through the coil causes radio-frequency electric and magnetic fields to be set up in the area at the top of the torch. When a spark (produced by a Tesla tube or some other seeding device) is applied to the support gas flowing through the torch, some electrons are stripped from the support gas atoms. These electrons are then caught up in the magnetic field and accelerated. Adding energy to the electrons by the use of a coil is known as inductive coupling. These high-energy electrons in turn collide with other support-gas atoms, stripping off still more electrons. The collisional ionisation of the support gas continues in a chain reaction, breaking down the gas into a physical plasma consisting of support-gas atoms, electrons and support-gas ions. The plasma is then sustained within the torch and load coil as radio-frequency energy is continually transferred to it through the inductive coupling process.

The ICP appears as an intense, very bright, plume-shaped plasma. At the base the plasma is toroidal, and this is referred to as the induction region (IR), i.e. the region in which the inductive energy transfer from the load coil to the plasma takes place. The sample is introduced through the induction region into the centre of the plasma.

Apparatus

ICP-AES consists of sample-introduction system with peristaltic pump for delivering the solution at constant flow rate into a nebuliser, radio-frequency (RF) generator, plasma torch, transfer optics focussing the image of the plasma at the entrance slit of the spectrometer, radial viewing is better for difficult matrices (alkalis, organics), whereas axial viewing gives more intensity and better detection limits in simple matrices, wavelength dispersive devices consisting of diffraction gratings, prisms, filters or interferometers, detectors converting radiant energy into electrical energy, data-acquisition unit.

Interference

Interference is anything that causes the signal from an analyte in a sample to be different from the signal for the same concentration of that analyte in a calibration solution. The well-known chemical interference that is encountered in flame atomic absorption spectrometry is usually weak in ICP-AES. In rare cases where interference occurs, it may be necessary to increase the RF power or to reduce the inner support-gas flow to eliminate it. The interference in ICP-AES can be of spectral origin or even the result of high concentrations of certain elements or matrix compounds. Physical interference (due to differences in viscosity and surface tension of the sample and calibration standards) can be minimised by dilution of the sample, matrix matching and use of internal standards or through application of the method of standard additions.

Another type of interference occasionally encountered in ICP-AES is the so-called 'easily ionised elements (EIEs) effect'. The EIEs are those elements that are ionised much more easily, for example alkaline metals and alkaline earths. In samples that contain high concentrations of EIEs (more than 0.1 per cent), suppression or enhancement of emission signals is likely to occur.

Spectral interference

This may be due to other lines or shifts in background intensity. These lines may correspond to argon (observed above 300 nm), OH bands due to the decomposition of water (at about 300 nm), NO bands due to the interaction of the plasma with the ambient air (between 200 nm and 300 nm), and other elements in the sample, especially those present at high concentrations. The interference falls into 4 different categories: simple background shift, sloping background shift, direct spectral overlap, and complex background shift.

Absorption interference

This arises when part of the emission from an analyte is absorbed before it reaches the detector. This effect is observed particularly when the concentration of a strongly emitting element is so high that the atoms or ions of that element that are in the lower energy state of transition absorb significant amounts of the radiation emitted by the relevant excited species. This effect, known as self-absorption, determines the upper end of the linear working range for a given emission line.

Multicomponent spectral fitting

Multiple emission-line determinations are commonly used to overcome problems with spectral interferences. A better, more accurate method for performing spectral interference corrections is to use the information obtained with advanced detector systems through multicomponent spectral fitting. This quantifies not only the interference, but also the background contribution from the matrix, thereby creating a correction formula. Multicomponent spectral fitting utilises a multiple linear-squares model based on the analysis of pure analyte,

the matrix and the blank, creating an interference-corrected mathematical model. This permits the determination of the analyte emission in a complex matrix with improved detection limits and accuracy.

Procedure

Sample preparation and sample introduction

The basic goal for the sample preparation is to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulphuric and phosphoric acids can contribute to background emission observed in the ICP spectra. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps used for ICP-AES usually deliver the standard and sample solutions at a rate of 1 ml per minute or less.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

Operating conditions

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen as wavelength selection, support-gas flow rates (outer, intermediate and inner tubes of the torch), RF power, viewing position (radial or axial), pump speed, conditions for the detector (gain/voltage for photomultiplier tube detectors, others for array detectors) and integration time (time set to measure the emission intensity at each wavelength).

Control of instrument performance

System suitability

The following tests may be carried out with a multi-element control solution to ensure the adequate performance of the ICP-AES system:

Energy transfer (generator, torch, plasma); measurement of the ratio Mg II (280.270 nm)/Mg I (285.213 nm) may be used, sample transfer, by checking nebuliser efficiency and stability; resolution (optical system), by measuring peak widths at half height, for example As (189.042 nm), Mn (257.610 nm), Cu (324.754 nm) or Ba (455.403 nm), analytical performance, by calculating detection limits of selected elements over the wavelength range.

Method validation

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

Linearity

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates. The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when the correlation coefficient is at least 0.99, the residuals of each calibration level are randomly distributed around the calibration curve. Calculate the mean and relative standard deviation for the lowest and for the highest calibration level. When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or more than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

Accuracy

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside of the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

Repeatability

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

Limit of quantification

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

2.4.43 Characterisation of Crystalline and Partially crystalline Solids by X-ray Powder Diffraction

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern.

Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially 3 types of information can be derived from a powder diffraction pattern: angular position of diffraction lines (depending on geometry and size of the unit cell); intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample); and diffraction line profiles (depending on instrumental resolution, crystallite size, strain and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (for example, identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually non-destructive in nature (specimen preparation is usually limited to grinding to ensure a randomly oriented sample. XRPD investigations can also be carried out under in situ conditions on specimens exposed to non-ambient conditions, such as low or high temperature and humidity.

Principle

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on the atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between 2 diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (Fig. 2.4.53.-1).

The wavelength λ of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or dhkl (also called 'd-spacings'). θhkl is the angle between the incident ray and the family of lattice planes, and sinθhkl is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices {hkl}. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings a, b and c and the angles between them, α, β, and γ.

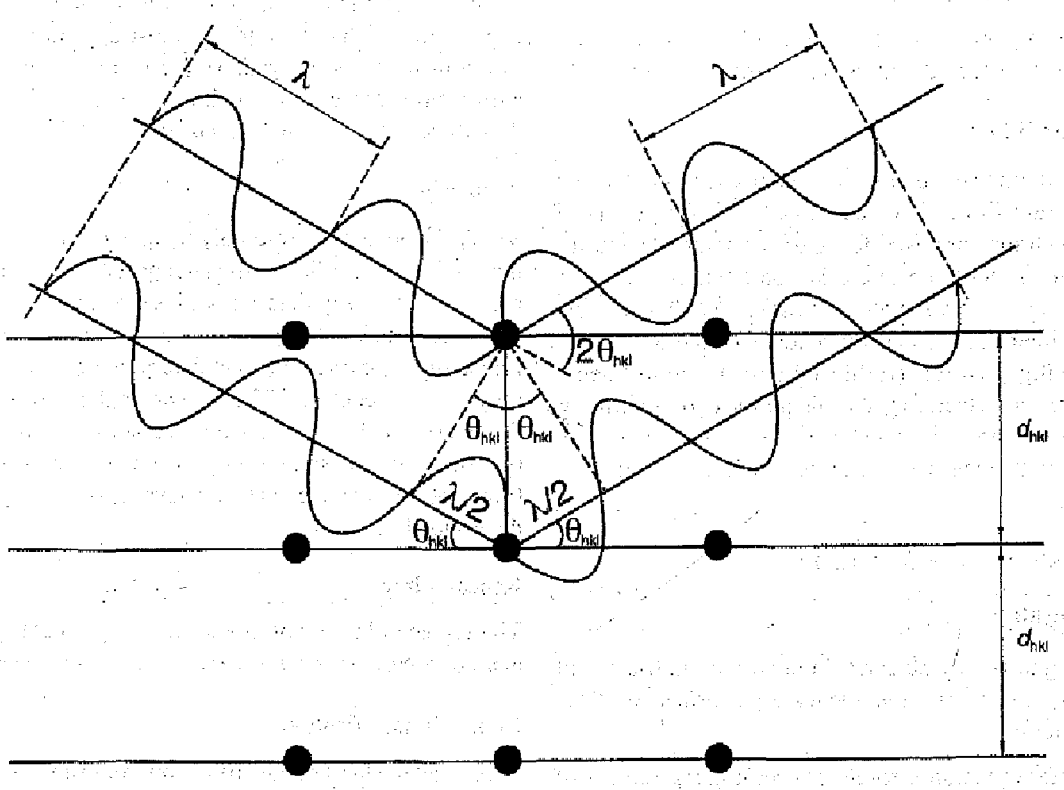


Fig. 2.4.53.-1. – *Diffraction of X-rays by a crystal according to Bragg's law*

The interplanar spacing for a specified set of parallel hkl planes is denoted by dhkl. Each such family of planes may show higher orders of diffraction where the d values for the related families of planes nh, nk, nl are diminished by the factor 1/n (n being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, θhkl, associated with it (for a specific wavelength λ).

A powder specimen is assumed to be polycrystalline so that at any angle θhkl there are always crystallites in an orientation allowing diffraction according to Bragg's law. For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as 'lines', 'reflections' or 'Bragg reflections') are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles on the perfection and extent of the crystal lattice. Under these conditions the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarisation factor, multiplicity and Lorentz factor.

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area and shape (characterised by, for example, peak width or asymmetry, analytical function, empirical representation). An example of the type of powder patterns obtained for 5 different solid phases of a substance are shown in Fig. 2.4.53.-2.

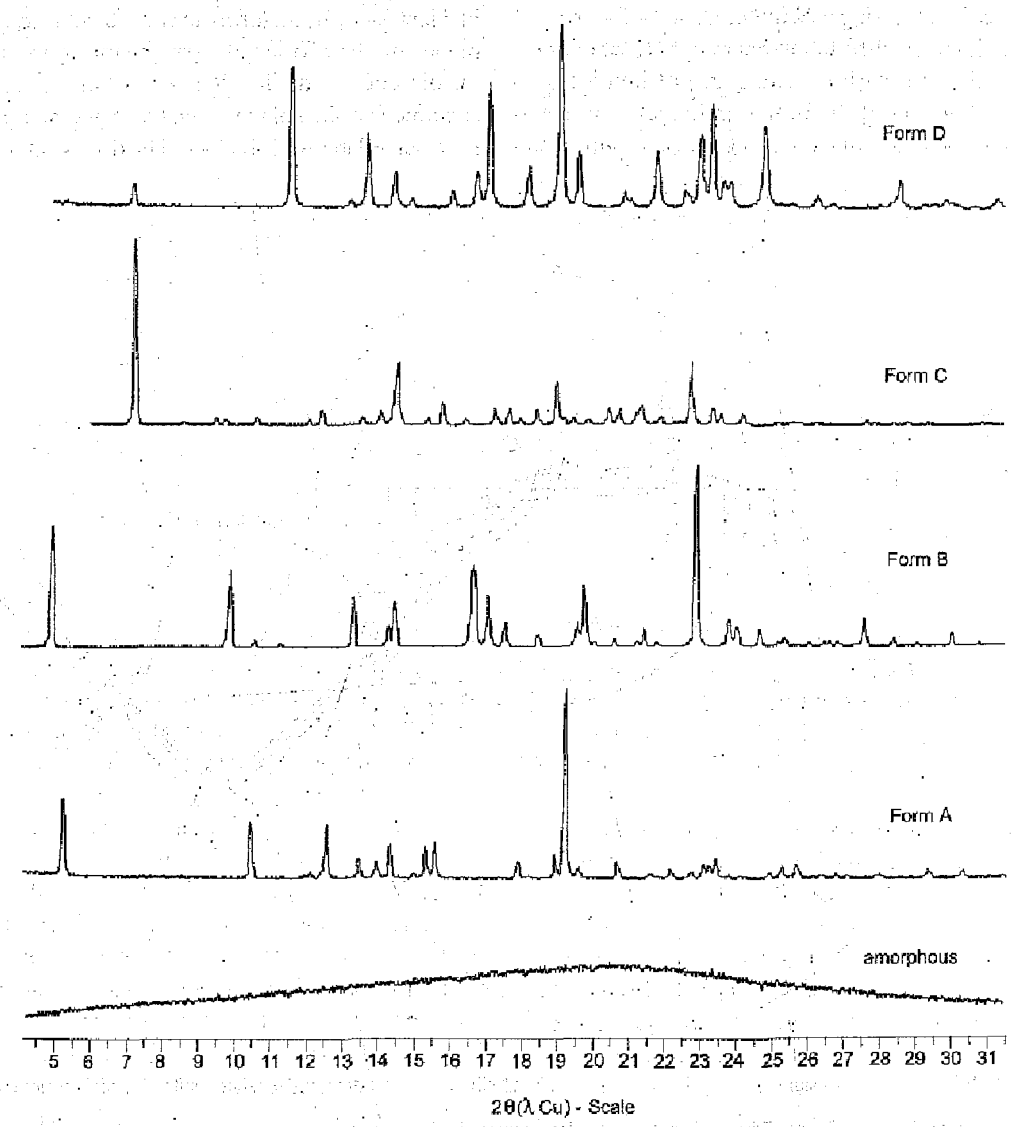


Fig. 2.4.53-2: X-ray powder diffraction patterns collected for five different solid phases of a substance (the intensities are normalized)

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more-or-less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background, for instance the sample holder, diffuse scattering from air and equipment, other instrumental parameters such as detector noise, general radiation from the X-ray tube, etc. The peak-to-background ratio can be increased by minimising background and by choosing prolonged exposure times.

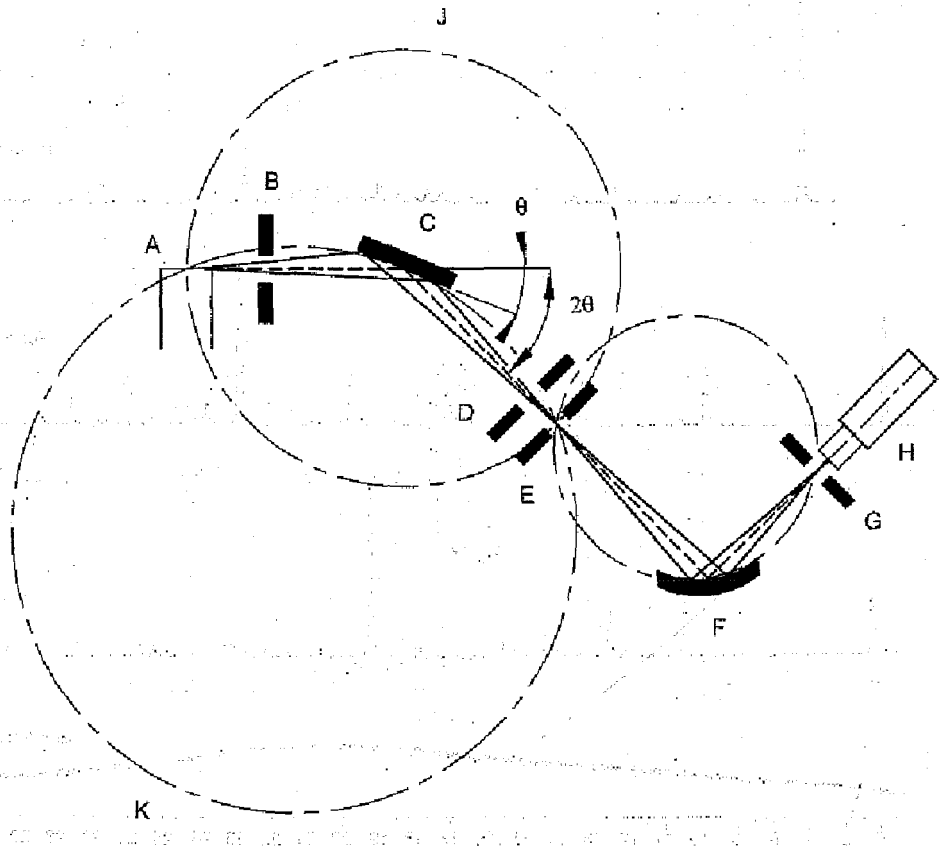
Instrument

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises 5 main parts: an X-ray source; incident beam optics, which may perform monochromatisation, filtering, collimation and/or focusing of the beam; a goniometer; diffraction beam optics, which may perform monochromatisation, filtering, collimation and focusing or parallelising of the beam; and a detector. Data-collection and data-processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure XRPD patterns are powder cameras. The replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing but parafocusing, such as in the Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used.

A given instrument may provide a horizontal or vertical θ/2θ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane). The basic geometric arrangement is represented in Fig. 2.4.53.-3 . The divergent beam of radiation



A. X-ray tube C. sample E. receiving slit G. detector receiving slit J. diffractometer circle

B. divergence slit D. anti-diffusion slit F. monochromator H. detector R. focusing circle

Figure 2.4.53.-3: Geometric arrangement of the Bragg-Brentano parafocusing geometry

from the X-ray tube (the so-called 'primary beam') passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For θ/2θ scans the goniometer rotates the specimen about the same axis as that of the detector, but at half the rotational speed, in a θ/2θ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5-2 mm thickness can also be used for small sample amounts.

X-ray radiation. In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources utilised for X-ray diffraction are vacuum tubes utilising copper, molybdenum, iron, cobalt or chromium as anodes: copper, molybdenum or cobalt X-rays are employed most commonly for organic substances (the use of cobalt anodes can be especially preferred to separate distinct X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the Kα radiation from the anode. Consequently, it is advantageous to make the X-ray beam 'monochromatic' by eliminating all the other components of the emission spectrum. This can be partly obtained using Kβ filters, i.e. metal filters selected as having an absorption edge between the Kα and Kβ wavelengths emitted by the tube.

Such a filter is usually inserted between the X-ray tube and the specimen. Another, more-and-more-commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a 'monochromator'). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e. Kα and Kβ) at different angles, so that only one of them may be selected to enter into the detector. It is even possible to separate Kα1 and Kα2 radiations by using a specialised monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating Kα and Kβ wavelengths is by using curved X-rays mirrors that can simultaneously monochromate and focus or parallelise the X-ray beam.

Radiation Protection- Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions are taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are established.

Specimen Preparation and Mounting

The preparation of the powdered material and mounting of the specimen in a suitable holder are critical steps in many analytical methods, and are particularly so for XPRD analysis, since they can greatly affect the quality of the data to be collected. The main sources of error due to specimen preparation and mounting are briefly explained in in Bragg-Brentano para focusing geometry.

Specimen Preparation

The morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or plate-like crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections, so that some are more intense and others are less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimise preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 μm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 μm) may

cause line broadening and significant changes to the sample itself such as:

- specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.);
- reduced degree of crystallinity;
- solid-state transition to another polymorph;
- chemical decomposition;
- introduction of internal stress;
- solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the non-ground specimen with that corresponding to a specimen of smaller particle size (e.g. a milled specimen). If theXRPD pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

If a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

Specimen Mounting

Effect of specimen displacement. A specimen surface that is offset by D with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D \cdot \cos\theta$ shifts in 2θ positions (typically of the order of 0.01° in 2θ at low angles ($\cos\theta \approx 1$) for a displacement D = 15 μm) and asymmetric broadening of the profile towards low 2θ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This is by far the largest source of errors in data collected on well-aligned diffractometers.

Effect of specimen thickness and transparency. When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of 'infinite thickness'. To minimize the transparency effect, it is advisable to use a non-diffracting substrate (zero background holder), for example a plate of single crystalline silicon cut parallel to the 510 lattice planes. One advantage of the transmission mode is that problems with sample height and specimen transparency are less important. The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

Control of the Instrument Performance

Goniometers and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to minimise adequately systematic errors, while optimising the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought whilst performing the alignment procedure. There are many different configurations and each supplier's equipment requires specific alignment procedures.

The overall diffractometer performance must be tested and monitored periodically using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

Qualitative Phase Analysis (Identification of Phases)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its XRPD pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterised single-phase specimens. It possible in most cases to identify a crystalline substance by its 2θ diffraction angles or d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based either on a more-or-less extended 2θ-range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalised intensities (I_{norm}), a so-called (d, I_{norm})-list extracted from the pattern, is the crystallographic fingerprint of the material, and can be compared to (d, I_{norm})-lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu K_α radiation, it is appropriate to record the diffraction pattern in a 2θ-range from as near 0° as possible to at least 40°. The agreement in the 2θ-diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognised to have varying unit cell dimensions and as such shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ-positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g. inorganic salts), it may be necessary to extend the 2θ-region scanned to well beyond 40°. It is generally sufficient to scan past the 10 strongest reflections identified in single phase XRPD database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- non-crystallised or amorphous substances;
- the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10 per cent *m/m*);

- pronounced preferred orientation effects,
- the phase has not been filed in the database used,
- formation of solid solutions,
- presence of disordered structures that alter the unit cell,
- the specimen comprises too many phases,
- presence of lattice deformations,
- structural similarity of different phases.

Quantitative Phase Analysis

If the sample under investigation is a mixture of 2 or more known phases, of which not more than 1 is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can, in many cases, be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines or on the full pattern. These integrated intensities, peak heights or full-pattern data points are compared to the corresponding values of reference materials. These reference materials shall be single-phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require in particular homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects. In favourable cases, amounts of crystalline phases as small as 10 per cent may be determined in solid matrices.

Polymorphic Samples

For a sample composed of 2 polymorphic phases a and b, the following expression may be used to quantify the fraction F_a of phase a:

$$F_a = \frac{1}{1 + K(I_b / I_a)}$$

The fraction is derived by measuring the intensity ratio between the 2 phases, knowing the value of the constant K. K is the ratio of the absolute intensities of the 2 pure polymorphic phases I_{oa}/I_{ob}. Its value can be determined by measuring standard samples.

Methods using a Standard

The most commonly used methods for quantitative analysis are:

- the 'external standard method';
- the 'internal standard method';
- the 'spiking method' (often also called the 'standard addition method').

The 'external standard method' is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material with crystallite size and X-ray absorption coefficient comparable to those of the components of the sample, and with a diffraction pattern that does not overlap at all that of the sample to be analysed, can be used. A known quantity of this reference material is added to the sample to be analysed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the 'internal standard method', requires a precise measurement of diffraction intensities.

In the 'spiking method' (or 'standard addition method'), some of the pure phase a is added to the mixture containing the unknown concentration of a. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative x intercept is the concentration of the phase a in the original sample.

Estimate of the Amorphous and Crystalline Fractions

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:

- If the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances as described above; the amorphous fraction is then deduced indirectly by subtraction;
- If the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase ('the degree of crystallinity') can be estimated by measuring 3 areas of the diffractogram:

A = total area of the peaks arising from diffraction from the crystalline fraction of the sample;

B = total area below area A;

C = background area (due to air scattering, fluorescence, equipment, etc).

When these areas have been measured, the degree of crystallinity can be roughly estimated using the following formula:

$$\text{Per cent crystallinity} = 100A/(A + B - C)$$

It is noteworthy that this method does not yield absolute degree-of-crystallinity values and hence is generally used for comparative purposes only.

More sophisticated methods are also available, such as the Ruland method.

Single Crystal Structure

The determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low and the scattering properties are normally very low.

For any given crystalline form of a substance, knowledge of the crystal structure allows the calculation of the corresponding XRPD pattern, thereby providing a 'preferred-orientation-free' reference XRPD pattern, which may be used for phase identification.

2.4.44. Flash Point

The flash point of a volatile material is the lowest temperature at which it can vaporize to form an ignitable mixture in air. At the flash point, the vapour may cease to burn when the source of ignition is removed. The flash point is often used as a descriptive characteristic of liquid fuel, and it is also used to help characterize the fire hazards of liquids. "Flash point" refers to both flammable liquids and combustible liquids.

The flash point test method is a dynamic test method and depends on definite rates of temperature increases to control the precision of the test method. The rate of heating may not in all cases give the precision quoted in the test method because of the low thermal conductivity of certain materials.

Flash point values are a function of the apparatus design, the condition of the apparatus used, and

the operational procedure carried out. Flash point can therefore only be defined in terms of a standard test method, and no general valid correlation can be guaranteed between results obtained by different test methods, or with test apparatus different from that specified.

The following procedures ars suitable for the various substances described in the Pharmacopoeia.

Any other apparatus may also be used. The accuracy should be checked frequently by using certified reference substances of declared flash point.

Apparatus. Pensky Martens closed cup apparatus

The apparatus may be manual or automated. It consists of test cup, test cover and shutter stirring device, heating source, ignition source device, air bath, and top plate.

The accuracy and precision of flash point measurements can be increased if the following precautions are taken.

a) Support the manual or automated apparatus on a level steady surface, such as a table.

b) Tests are to be performed in a draft-free room or compartment. Tests made in a laboratory hood or in any location where drafts occur are not reliable.

c) Prepare the manual apparatus or the automated apparatus for operation in accordance with the manufacturer's instructions for calibrating, checking, and operating the equipment.

d) Thoroughly clean and dry all parts of the test cup and its accessories before starting the test, to ensure the removal of any solvent which had been used to clean the apparatus. Use suitable solvent capable of removing all of the specimen from the test cup and drying the test cup and cover.

Method

a) Fill the test cup with the test specimen to the filling mark inside of the test cup. The temperature of the test cup and test specimen shall be at least 18°C or 32°F below the expected flash point.

b) Place the test cover on the test cup and place the assembly into the apparatus. Be sure the locating or locking device is properly engaged. If the temperature measuring device is not already in place, insert the device into its holder.

c) Light the test flame, and adjust it to a diameter of 3.2 to 4.8 mm (0.126 to 0.189 in.), or switch on the electric igniter and adjust the intensity in accordance with the manufacturer's instructions.

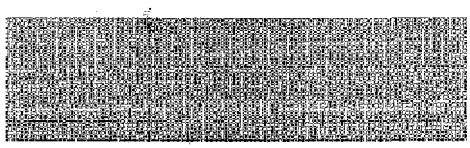
d) Apply the heat at such a rate that the temperature, as indicated by the temperature measuring device, increases 5° to 6° per minute.

e) Turn the stirring device at 90 to 120 rpm, stirring in a downward direction. If the test specimen is expected to have a flash point of 110° or below, apply the ignition source when the temperature of the test specimen is 23 ± 5° below the expected flash point and each time thereafter at a temperature reading that is a multiple of 1°. Discontinue the stirring of the test specimen and apply the ignition source by operating the mechanism on the test cover which controls the shutter so that the ignition source is lowered into the vapor space of the test cup in 0.5 s, left in its lowered position for 1 s, and quickly raised to its upward position.

f) Record as the observed flash point the reading on the temperature measuring device at the time ignition source application causes a distinct flash in the interior of the test cup. The sample is deemed to have flashed when a large flame appears and instantaneously propagates itself over the entire surface of the test specimen.

g) When the ignition source is a test flame, the application of the test flame may cause a blue halo or an enlarged flame prior to the actual flash point. This is not a flash and shall be ignored.

h) When the apparatus has cooled down to a safe handling temperature, less than 55° remove the test cover and the test cup and clean the apparatus as recommended by the manufacturer.



2.4.45. Odour and Taste

The characteristics for taste and odour are indicated in many substances because they may be useful for descriptive properties of substances. However, they are not meant to be applied as tests for identifying materials.

The inclusion of odour and (or) taste among other descriptive properties may help in identifying causative agent following accidental exposure to or contact with the substance. This information is provided as a warning or to make an individual aware of sensations that may be encountered. The use of odour or taste as a test for identification or content is strongly discouraged.

The characteristic odour of a volatile substance becomes apparent immediately on opening of a container. It is advisable to open the containers of such substances in a well ventilated fume hood. The verification of odour should be carried out using a watch-glass 6 cm to 8 cm in diameter, spreading in a thin layer 0.5 g to 2.0 g of the substance to be examined. After 15 min, determine the odour or verify the absence of odour.

NOTE — where therapeutically potent drugs are to be evaluated like diabetes, anti-anxiety or oncology drugs, the test is to be avoided and alternate instrumental tests may be considered.

2.4.46. Approximate pH of Solutions

Determine the approximate pH using a *pH indicator test papers*. Alternatively, pH indicators such as those described in Table 1 can be used.

Table 1

Reaction	pH	Indicator
Alkaline	More than 8.0	Red litmus paper
Slightly alkaline	8.0 to 10.0	Phenolphthalein solution
		Thymol blue solution
Strongly alkaline	More than 10.0	Phenolphthalein Paper
		Thymol blue solution
Neutral	6.0 to 8.0	Methyl red solution
		Phenol red solution
Acid	Less than 6.0	Methyl red solution
		Bromothymol blue solution
Slightly acid	4.0 to 6.0	Methyl red solution
		Bromocresol green solution
Strongly acid	Less than 4.0	Congo red paper

2.4.47. Raman Spectrometry

In Raman Spectrometry a sample is irradiated with an intense monochromatic light source (usually a laser). Most of the radiation is scattered from the sample at the same wavelength as that of the incident light; this process is known as Rayleigh scattering or elastic light scattering. Only 10^{-6} to 10^{-8} of the incident photons are scattered from the sample at wavelengths shifted from the original source wavelength; this scattered light is known as Raman scatter or inelastic light scatter. The differences between the incident light source wavelength and the Raman scattered wavelengths are known as the Raman shifts and are related to the molecular vibrations within the sample. The scattered light with lower energy is called Stokes scatter and with higher energy is called anti-Stokes scatter. Typically, Stokes scatter is analysed.

A Raman spectrum can be obtained from liquid, solid and even gaseous samples. Raman Spectrometry is a suitable non-invasive method for determination of solid-state properties and for chemical identification, for example, detecting changes in polymorphic form. Raman Spectrometry encompasses many methodologies, including backscattering, transmittance, resonance Raman (RR) Spectrometry, surface enhanced Raman Spectrometry (SERS), tip-enhanced Raman Spectrometry (TERS), spatially offset Raman Spectrometry (SORS), Raman optical activity (ROA), coherent anti-Stokes Raman Spectrometry (CARS), stimulated Raman Spectrometry (SRS) and confocal (CF) Raman Spectrometry. It also lends itself to imaging techniques.

Raman Spectrometry is complementary to infrared Spectrometry. Both techniques probe the fundamental molecular vibrations in a substance. However, raman and infrared Spectrometry have different sensitivities for different functional groups within a substance. Raman Spectrometry is particularly useful in examining non-polar bonds, functional groups and vibrations that are highly symmetrical (e.g. C-C single or multiple bonds); but is less sensitive to polar bonds (e.g. C=O) and vibrations that are asymmetrical. For example, *water*, which strongly influences infrared spectra, exhibits weak raman scattering and therefore has minimal interference on the resulting spectrum. Raman can therefore be used for aqueous solutions.

A major difficulty of Raman Spectrometry is that the substance examined or impurities in the substance may exhibit fluorescence, which can overcome the Raman signal. Fluorescence may be avoided by choosing a longer excitation wavelength, for example in the near-infrared region.

Raman Spectrometry is a rapid and non-invasive analytical method and can be performed off-line and also at-line, on-line or in-line for process analytical technology (PAT). Raman spectrometers can be situated at a great distance from the point of measurement when using long-distance optical fibres

for sample interfacing. Raman Spectrometry has a wide variety of applications for chemical, physical and process analysis and can also be used for counterfeit detection and quality control, for example.

- *chemical analysis*: identification and quantification of active substances, excipients;
- *physical analysis*: identification and quantification of solid forms (e.g. polymorphs and solvates) and crystallinity;
- *process analysis*: monitoring of biological and chemical reactions, synthesis, crystallisation, granulation, mixing, drying, lyophilisation, extrusion, encapsulation and coating.

Apparatus

There are different types of spectrometers for recording Raman spectra, the most common are benchtop dispersive instruments, including microscope-coupled Raman spectrometers, Fourier transform (FT) and hand-held Raman spectrometers.

Raman spectrometers typically consist of the following components.

- a monochromatic light source, typically a laser, with a wavelength in the ultraviolet, visible or near-infrared region; selection of laser frequency and intensity should be considered depending on the intended application and substance under examination;
- suitable optics (lens, mirrors or optical-fibre assembly) that direct the monochromatic light to and collect the Raman scattered light from the substance under examination;
- an optical device (monochromator or filter) that transmits the frequency-shifted Raman scattering and prevents the intense incident frequency of Rayleigh scattering from reaching the detector; and
- a dispersing device (grating and/or prism polychromator) combined with wavelength-selecting slits and a single or multi-channel detector (e.g. a charge-coupled device) or
- an interferometer with a detector that records the intensity of the scattered light over time, and a data-handling device (e.g. computer and appropriate software) that converts this data to the frequency or wavenumber domain by a Fourier-transform calculation.

Preparation of the sample

Raman spectra can be obtained from solids, liquids and gases either directly or in suitable containers such as glass vials, generally without prior sample preparation or dilution.

When using Raman Spectrometry, the measured sample area and volume may be small (in particular for microscope-coupled devices) and care must be taken to ensure the measurement is representative. This can be achieved by, for example, rotation of the sample, performing multiple measurements on different preparations of the sample, increasing the area of illumination by reducing the magnification (focus), by demagnification (defocusing) of the laser beam or by changing the focal length between measurements to scan at different depths.

It is not always possible to consider Raman as a non-destructive technique. The energy transmitted by the laser depends on the duration of exposure and the wavelength. It may change the physical state and may destroy the sample.

Control of instrument performance

Carry out the prescribed calibrations and/or system performance tests according to the manufacturer's instructions at regular intervals, depending on the use of the apparatus. Wavenumber calibration of Fourier transform systems is maintained with an internal laser (He-Ne). For dispersive systems, emission spectra of a low pressure lamp exhibiting characteristic maxima at wavenumbers over the whole spectral range of the instrument can usually be used for calibration (e.g. neon lamps or less frequently mercury, argon, krypton or xenon lamps).

Verification of the wavenumber scale. Verify the wavenumber scale of the Raman shift using a suitable standard that has characteristic maxima at the wavenumbers under investigation, for example an organic substance such as polystyrene, paracetamol or cyclohexane (see Table 1).

Table 1– Wavenumber shifts (and acceptance tolerances) of Polystyrene, Paracetamol, and Cyclohexane

	Wavenumber Shifts[a] (cm^{-1})	Tolerances Benchtop (cm^{-1})	Tolerances Hand-held (cm^{-1})
Polystyrene[b]	620.9	±1.5	±2.5
	1001.4	±1.5	±2.0
	1031.8	±1.5	±2.0
	1602.3	±1.5	±3.0
	3054.3	±3.0	NA[c]
Paracetamol[c]	797.2	±1.5	±2.5
	857.9	±1.5	±2.0
	1168.5	±1.5	±2.0
	1236.8	±1.5	±2.0
	1323.9	±1.5	±2.5
	1648.4	±1.5	±3.0
	2931.1	±2.0	NA[c]
Cyclohexane[d]	801.3	±1.5	±2.5
	1028.3	±1.0	±2.0
	1266.4	±1.0	±2.0
	1444.4	±1.0	±2.5
	2852.9	±2.0	±3.0

[a]Standard guide for Raman shift standards for spectrometer calibration (American Society for Testing and Materials ASTM E 1840),

[b]Polystyrene film (*e.g. 76 µm*), pellets (*e.g. NIST 706a*) *or rod*,

[c]Certified Paracetamol for calibration (which represents monoclinic form 1),

[d]*Cyclohexane*,

[e]NA: beyond detector range.

A minimum of 3 wavenumber shifts covering the working range of the instrument intended for measurements should be chosen. For dispersive Raman spectrometers that use multiple gratings for different spectral resolutions, the wavenumber scale should be verified at the same optical resolution that will occur for sample collection. All gratings used for Raman measurements should be verified for accuracy of Raman-shift.

Verification of the response-intensity scale. The absolute and relative intensities of the Raman bands are affected by variations in several factors including.

- polarisation of the irradiating light;
- polarisation of the Raman scattered light;
- intensity of the irradiating light;
- instrument response;
- focus and geometry at sample;
- packing density for solid samples;
- refractive index n or change of n (Δn) between analyte and the environment;
- the particle size and particle-size distribution;
- the scattering cross-section;
- the absorption cross-section.

Appropriate acceptance criteria will vary with the application but a day-to-day variation of ± 10 per cent or less in relative band intensities is achievable in most cases. Response calibration may involve the use of white-light standards or luminescent glass (e.g. NIST SRM 2241), usually applied by the manufacturer of Raman spectrometers.

Qualitative methods

Since the frequency shift position is employed for identification, identical laser intensity for both the reference standard and the substance under examination may not be necessary. The substance under examination is measured in the same physical state (e.g. liquid, solid) as the reference or library material. Raman techniques offer the advantage of non-invasive measurements of the substance under examination without removal from the packaging. However, some packaging materials may alter the measurement. This is especially the case when the packaging absorbs at the laser's excitation wavelength.

Identification using a reference standard. Prepare the substance under examination and the reference standard by the same procedure and record the spectra under the same instrumental conditions. The maxima in the spectrum obtained with the substance under examination correspond in position and if applicable in relative intensity to those in the spectrum obtained with the reference standard. When the spectra recorded in the solid state show differences in the positions of the maxima, treat the substance under examination and the reference standard in the same manner so that they crystallise or are produced in the same solid form, or proceed as described in the monograph, then record the spectra.

Identification using a spectral reference library. Record the spectra of a suitable number of materials which exhibit typical variation (*manufacturer, batch, particle size, impurity profile*, etc.) and comply with the requirements of the monograph or established specifications. The number and selection of samples in the database depends on the specific application. A suitable mathematical transformation of the Raman spectrum may facilitate qualitative comparison of spectra (e.g. baseline correction, min-max normalisation, vector normalisation, derivatives). The selectivity of the database that makes it possible to identify a given substance and distinguish it adequately from other substances in the database is to be confirmed during the validation procedure.

Quantitative methods

Quantitative determination requires that the reference standard and the substance under examination are measured at the same laser intensity and frequency. Ensure the substance under examination is measured in the same physical state (e.g. liquid, solid) and concentration range as the reference standard used for calibration. While Beer-Lambert's law is not valid for Raman Spectrometry, Raman intensity is directly proportional to the concentration of the Raman scattering analytes, however for solid samples and suspensions the Raman intensity could be affected by the matrix (e.g. fluorescence and self-absorption). The Raman signal is influenced by the refractive index of the substances, the particle size and the particle-size distribution (where the small particles give a relatively more intense Raman scattering than large particles), the packing density, the scattering cross-section, the absorption cross-section, etc. (see also under Verification of the response-intensity scale).

2.5. PHARMACEUTICAL METHODS

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2.5.1. Disintegration Test

This test determines whether dosage forms such as tablets, capsules, boluses pessaries and suppositories disintegrate within a prescribed time when placed in a liquid medium under the prescribed experimental conditions.

For the purpose of this test, disintegration does not imply complete solution of the dosage unit or even of its active constituent. Disintegration is defined as that state in which no residue of the unit under test remains on the screen of the apparatus or, if a residue remains, it consists of fragments of disintegrated parts of tablets component parts such as insoluble coating of the tablets or of capsule shells, or of any melted fatty substance from the pessary or suppository or is a soft mass with no palpable core. If discs have been used with capsules, any residue remaining on the lower surfaces of the discs consists only of fragments of shells.

NOTE — Use apparatus A for tablets and capsules that are not more than 18 mm long. For larger tablets and capsules use apparatus B.

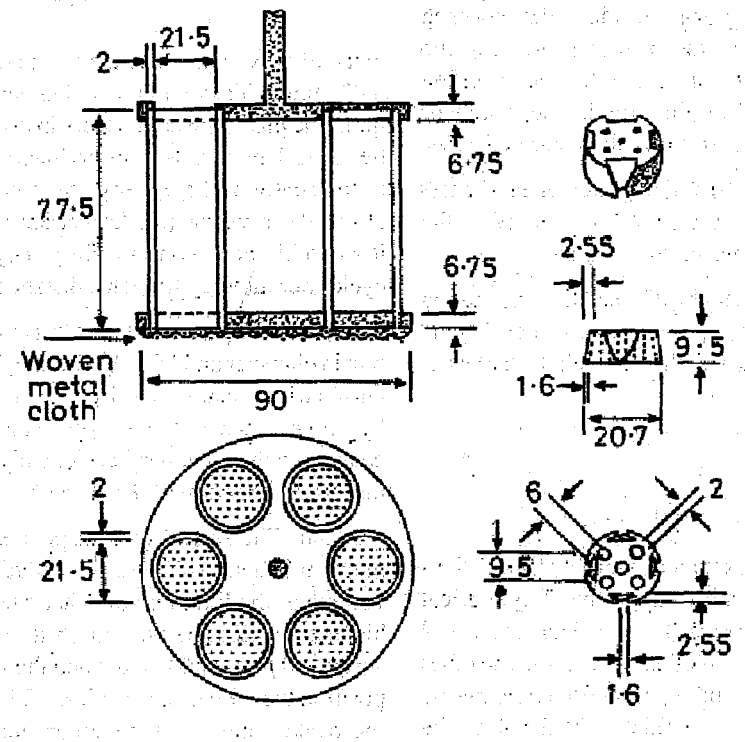
Apparatus A

For tablets, boltues and capsules of normal size

The apparatus consists of a basket-rack assembly, a 1-litre beaker, a thermostatic arrangement for heating the fluid and a mechanical device for raising and lowering the basket in the immersion fluid at a constant frequency rate.

Basket-rack assembly. The basket-rack assembly is rigid and supports six cylindrical glass tubes, 77.5 ± 2.5 mm long, 21.5 mm in internal diameter and with a wall thickness of about 2 mm (Fig. 2.5.1-1). The tubes are held vertically by two superimposed transparent plastic plates, 90 ± 2 mm in diameter and 6.75 ± 1.75 mm thick perforated by six holes having the same diameter as the tubes. The holes are equidistant from the centre of the plate and are equally spaced from one another. Attached to the under side of the lower plate is a woven stainless steel wire cloth with a plain square weave with 2.0 ± 0.2 mm mesh apertures and with a wire diameter of 0.615 ± 0.045 mm. The upper plate is covered with a stainless steel disc perforated by six holes, each about 24 ± 2 mm in diameter, which fits over the tubes and holds them between the plastic plates. The holes coincide with those of the upper plastic plate and the upper open ends of the glass tubes. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery and a metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached to the device for raising and lowering it smoothly at a constant frequency of between 29 and 32 cycles per minute through a distance of 55±2 mm. The time required for the



(Dimensions in mm)

Fig. 2.5.1-1: Apparatus for Disintegration of Tablets and Capsules

upward stroke is equal to the time required for the downward stroke, and the change in stroke direction should be smooth and not abrupt. There should be no appreciable horizontal motion or movement of the axis from the vertical.

The design of the basket-rack assembly may be somewhat provided with different specifications for the glass tubes and the screen mesh size are unchanged.

Disc. A cylindrical disc for each tube, each 20.7 ± 0.15 mm thick in diameter and 9.5 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18 to 1.20, and pierced with five holes, each 2 mm in diameter, one in the centre and the other four spaced equally on a circle of radius 6 mm from the centre of the disc. Four equally-spaced grooves are cut in the lateral surface of the disc in such a way that at the upper surface of the disc they are 9.5 mm wide and 2.55 mm deep and at the lower surface 1.6 mm.

Medium. The assembly is suspended in the liquid medium in a suitable vessel, preferably a 1-litre beaker. The volume of liquid is such that the wire mesh at its highest point is at least 15 mm below the surface of the liquid, and at its lower point is at least 25 mm above the bottom of the beaker. At no time should the top of the basket-rack assembly become submerged. There is a thermostatic arrangement for heating the liquid and maintaining the temperature at 37° ± 2°.

Method. Unless otherwise stated in the individual monograph, introduce one tablet or capsule into each tube and, if directed in the appropriate general monograph, add a disc to each tube. Suspend the assembly in the beaker containing the specified liquid and operate the apparatus for the specified time. Remove the assembly from the liquid. The tablets or capsules pass the test if all of them have disintegrated.

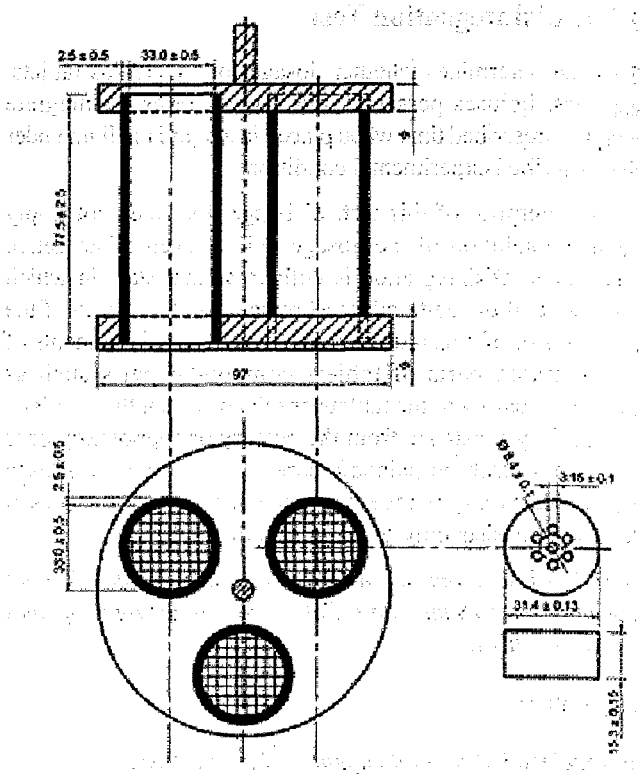
If 1 or 2 tablets or capsules fail to disintegrate, repeat the test on 12 additional tablets or capsules; not less than 16 of the total of 18 tablets or capsules tested disintegrate.

If the tablets or capsules adhere to the disc and the preparation under examination fails to comply, repeat the test omitting the disc. The preparation complies with the test if all the tablets or capsules in the repeat test disintegrate.

Apparatus B

For tablets, boluses and capsules of large size

Basket rack assembly. The Basket rack assembly (Fig. 2.5.1.-2.) is a rigid basket- rack assembly supporting 3 cylindrical transparent tubes 77.5 ± 2.5 mm long, 33.0 ± 0.5 mm in internal diameter, and with a wall thickness of 2.5 ± 0.5 mm. The tubes are held vertically by 2 separate and superimposed rigid plastic plates 97 mm in diameter and 9 mm thick, with 3 holes. The holes are equidistant from the centre of the plate and equally spaced. Attached to the under side of the lower plate is a



(Dimensions in mm)

Fig. 2.5.1-2: Apparatus for Disintegration of Tablets and capsules of large size

piece of woven gauze made from stainless steel wire 0.63 ± 0.03 mm in diameter and having mesh apertures of 2.0 ± 0.2 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery. A metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached to a mechanical device capable of raising and lowering it smoothly at a constant frequency of between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm.

Disc. Each tube is provided with a cylindrical disc 31.4 ± 0.13 mm in diameter and 15.3 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18-1.20. Each disc is pierced by 7 holes, each 3.15 ± 0.1 mm in diameter, One hole is in the centre and the other 6 spaced equally on a circle of radius 4.2 mm from the centre of the disc.

Medium. The assembly is suspended in the specified liquid medium in a suitable vessel, preferably a 1-liter beaker. The volume of the liquid is such that when the assembly is in the highest position the wire mesh is at least 15 mm below the surface of the liquid, and when the assembly is in the lowest position the wire mesh is at least 25 mm above the bottom of the beaker and the upper open ends of the tubes remain above the surface of the liquid. A suitable device maintains the temperature of the liquid at 35-39°.

The design of the basket-rack assembly may be varied provided the specifications for the tubes and wire mesh are maintained.

Method. Unless otherwise stated in the individual monograph, introduce one tablet or capsule into each of the three tubes and, if directed in the appropriate general monograph, add a disc to each tube. Suspend the assembly in the beaker containing the specified liquid and operate the apparatus for the specified time, Remove the assembly from the liquid. The tablets or capsules pass the test if all six have disintegrated.

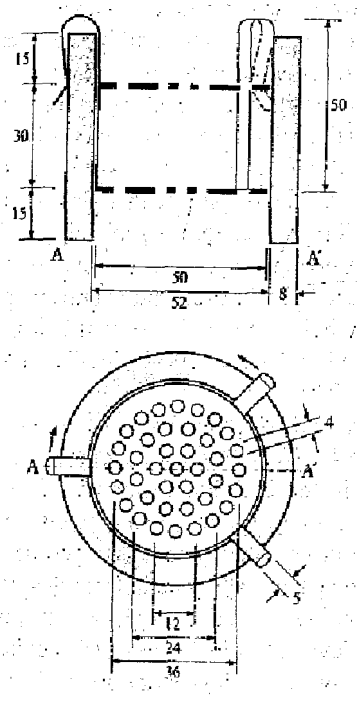
NOTE— Test six tablets or capsules either by using 2 basket-rack assemblies in parallel or by repeating the procedure.

Apparatus

For pessaries and suppositories

a) A transparent sleeve of glass or plastic, 60 mm high with an internal diameter of 52 mm and an appropriate wall thickness (Fig. 2.5.1-3a).

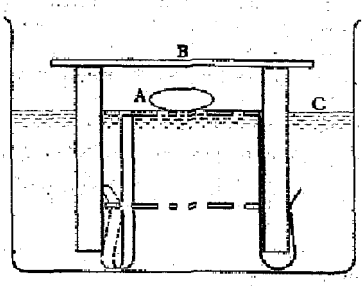
b) A metal device consisting of two stainless steel discs each of which contains 39 holes, each 4 mm in diameter, being distributed as indicated in Fig. 2.5.1-3a. The diameter of the disc is closely similar to the internal diameter of the sleeve. The discs are separated by a distance of about 30 mm. The metal device is attached to the outer sleeve by means of three equally spaced hooks.



(Dimensions in mm)

Fig. 2.5.1-3a: Apparatus for Disintegration of Pessaries and Suppositories

For Compressed Pessaries use with the hook-end downwards as in Fig. 2.5.1-3b.



A-Compressed pessary; B-Glass Plate; C-Water surfre

Fig. 2.5.1-3b: Apparatus for Disintegration of compressed pessaries

For Moulded Pessaries, Moulded Suppositories, Shell Pessaries and Shell Suppositories

Place a pessary or suppository on the lower perforated disc of the metal device and then insert the device into the cylinder and attach this to the sleeves. Repeat the operation with a further two pessaries or suppositories and metal devices and sleeves. Unless otherwise specified, place each piece of apparatus in a vessel containing at least 4 litres of water at 36° to 37° and fitted with a slow stirrer and a means of holding the top of the apparatus 90 mm below the surface of the water. A suitable thermostatic arrangement may be provided for maintaining the temperature of the bath. Alternatively, all three pieces of apparatus may be placed together in a vessel containing at least 12 litres of water. After each 10 minutes invert each apparatus without removing it from the liquid.

Disintegration is considered to be complete when the moulded pessary or suppository

a) is completely dissolved or

b) has dispersed into its component parts, which may remain on the surface (in the case of melted fatty substances), sink to the bottom (in case of insoluble powders) or dissolve (in case of soluble components) or may be distributed in one or more of these ways or

c) has become soft with appreciable change in shape, without necessarily separating into its components, and the mass has no solid core which cannot be pressed with a glass rod.

Apparatus

For Compressed Pessaries

Place the apparatus in a vessel of suitable diameter containing water at 36° to 37°. Adjust the level of the liquid by the gradual addition of water at 36° to 37° until the perforations in the

metal disc are just covered by a uniform layer of water. Place one compressed pessary on the upper perforated disc and cover the apparatus with a glass plate to ensure a humid atmosphere. Repeat the operation with a further two compressed pessaries.

Disintegration is considered to be complete when

a) There is no residue on the perforated plate or

b) If a residue remains, it consists only of a soft mass having no solid core which cannot be pressed with a glass rod.

2.5.2. Dissolution Test

This test is designed to determine compliance with the dissolution requirements for solid, semi-solid and suspension dosage forms.

Use Apparatus 2 (Paddle) unless otherwise directed. All parts of the apparatus that may come into contact with the preparation under examination or with the dissolution medium are chemically inert and do not adsorb, react or interfere with the preparation under examination. All metal parts of the apparatus that may come into contact with the preparation or the dissolution medium must be made from stainless steel, type 316 or equivalent or coated with a suitable material to ensure that such parts do not react or interfere with the preparation under examination or the dissolution medium.

No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation or vibration beyond that due to the smoothly rotating element.

An apparatus that permits observation of the preparation under examination and the stirrer during the test is preferable.

For dosage forms containing or coated with gelatin that do not conform to the dissolution specification, repeat the test as follows.

Dissolution Medium with pH 4.0 or less

Enzyme. Pepsin, activity determined by the Assay test in the monograph for Pepsin.

Amount. A quantity of pepsin that results in an activity of not more than 7,50,000 Units per litre of dissolution medium.

Dissolution Medium with more than pH 4.0 and less than 6.8

Enzyme. Papain, activity determined by the Assay test in the monograph for Papain or bromelain, activity determined by the procedure in bromelain under general reagents.

Amount. A quantity of papain that results in an activity of not more than 5,50,000 Units per litre of dissolution medium or a quantity of bromelain that results in an activity of not more than 30 gelatin–digesting units (GDU) per litre of dissolution medium.

Dissolution Medium with pH 6.8 or more

Enzyme. Pancreatin, protease activity determined by the procedure in Assay for protease activity in the monograph for Pancreatin.

Amount. A quantity of pancreatin that results in a protease activity of not more than 2000 Units per litre of dissolution medium.

NOTE — Appropriate organic solvent(s) may be used to enhance drug solubility for the preparation of the reference standard solutions. However, not more than 5 per cent (v/v) of the organic solvent should be present in the final solution unless validated.

Apparatus 1 (Basket Apparatus)

An assembly consisting of the following:

a. A cylindrical vessel, A, made of borosilicate glass or any other suitable transparent material, with a hemispherical bottom and with a nominal capacity of 1000 ml unless otherwise specified in the individual monograph and an inside diameter of 98-106 mm (Fig.2.5.2-1). The vessel has a flanged upper rim and is fitted with a lid that has a number of openings, one of which is central. Modified peak vessels can be used to eliminate coning. In addition to vessel parameters like inner diameter and height, evaluate parameters like hemisphere radius (49 mm to 53 mm).

b. A motor with a speed regulator capable of maintaining the speed of rotation of the serialized basket within 4 per cent of that specified in the individual monograph. The motor is fitted with a serialised basket, B (see Fig.2.5.2-2). The metallic shaft rotates smoothly and without significant wobble. The basket consists of two components. The top part, with a vent, is attached to the shaft C, it is fitted with three spring clips, or other suitable means like o-rings, that allow removal of the lower part for introduction of the preparation under examination and that firmly hold the lower part of the basket concentric with the axis of the vessel during rotation. The lower detachable part of the basket is made of welded-steam cloth, with a wire thickness of 0.265 ±0.045 mm diameter and with 0.4 ±0.04 mm square openings unless otherwise specified in the individual monograph, formed into a cylinder with narrow rim of sheet metal around the top and the bottom. Shaft and basket components of the stirring element are fabricated of stainless steel, type 316 or any other inert material. The basket may be plated with a 2.5 µm layer of gold for use with acidic media. The distance between the inside bottom of the vessel and the basket is maintained at 23 to 27 mm during the test. Evaluate the basket mesh integrity by using a magnifying glass or microscope at regular intervals.

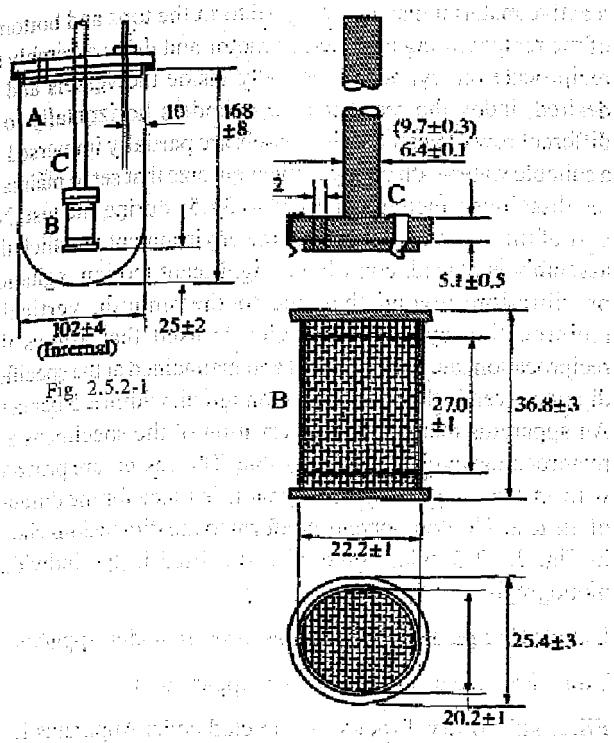


Fig. 2.5.2-1

(Dimensions in mm)

Fig. 2.5.2-2

c. A water-bath or any other suitable heating device, such as a heating jacket set to maintain the dissolution medium at 36.5° to 37.5°. The bath liquid is kept in constant and smooth motion during the test. The vessel is securely clamped in the water-bath in such a way that the displacement vibration from other equipment, including the water circulation device, is minimised.

Dissolution medium. Use the dissolution medium specified in the individual monograph. If the medium is a buffered solution, adjust the solution so that its pH is within 0.05 units of the pH specified in the monograph. The dissolution medium should be deaerated prior to testing.

Time. Where a single time specification is given in the monograph, the test may be concluded in a shorter period if the requirement for the minimum amount dissolved is met. Samples are to be withdrawn only at the stated times, within a tolerance of ± 2 per cent.

Filter suitability. It may be accomplished by preparing a suitable standard solution or a completely dissolved sample solution (e.g., prepared as a typical sample in a vessel or a sample put in a beaker and stirred with a magnetic stirrer for 1 hour). For standard solution, compare the results for filtered solution (after discarding the appropriate volume) to those for the unfiltered solution. For sample solution, compare the results for filtered solution (after discarding the appropriate volume) to those for centrifuged, unfiltered solution.

Apparatus 2 (Paddle Apparatus)

The assembly is the same as in Apparatus 1 except that the stirring element consists of a drive shaft and blade forming a paddle, D (Fig.2.5.2-3 and Fig. 2.5.2-4) The blade passes through the diameter of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The shaft is positioned so that its axis is within 2 mm of the axis of the vessel and the lower edge of the blade is 23 to 27 mm from the inside bottom of the vessel. The apparatus operates in such a way that the paddle rotates smoothly and without significant wobble.

A water-bath or any other suitable heating device, such as a heating jacket set to maintain the dissolution medium at 36.5° to 37.5°. The bath liquid is kept in constant and smooth motion during the test. The vessel is secured in the water-bath in such a way that the displacement vibration from other equipment, including the water circulation device, is minimised.

Dissolution medium. Proceed as directed under Apparatus 1.

Time. Proceed as directed under Apparatus 1.

Filter suitability. Proceed as directed under Apparatus 1.

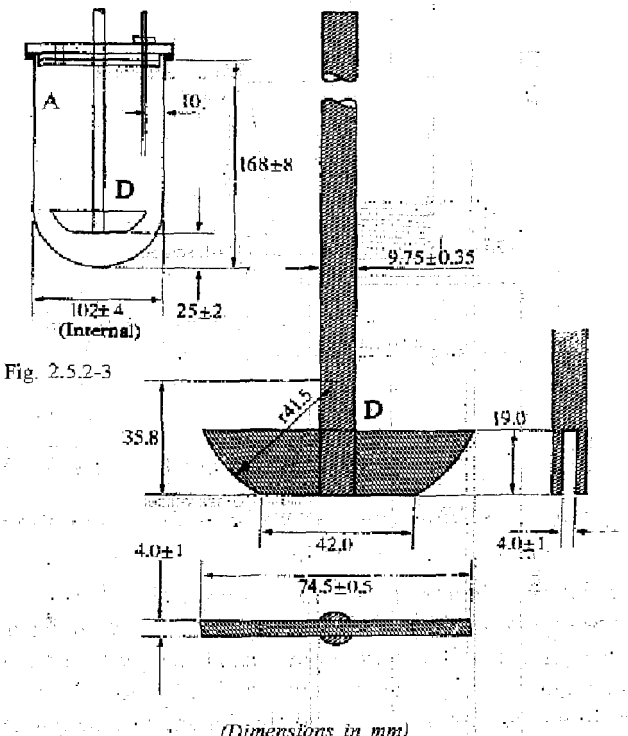


Fig. 2.5.2-3

(Dimensions in mm)

Fig. 2.5.2-4

Apparatus 3 (Reciprocating Cylinder)

The assembly consists of a set of cylindrical, flat-bottomed glass vessels, a set of glass reciprocating cylinders, inert fittings made from stainless steel, type 316 or equivalent or

coated with a suitable material to ensure that such parts do not react or interfere with the preparation under examination or the dissolution medium, screens (for eg. 20 mesh to 100 mesh) that are made of suitable non-absorbing and non-reactive material that are designed to fit the tops and bottoms of the reciprocating cylinders, a motor and drive assembly to reciprocate the cylinders vertically inside the vessels and if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water-bath of any convenient size that set to maintain the dissolution medium at 36.5° to 37.5° during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be selected and maintained at the specified dip rate given in the individual monograph within ± 5 per cent. An apparatus that permits observation of the specimens and reciprocating cylinders is preferable. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The components conform to the dimensions shown in Fig. 2.5.2-5 unless otherwise specified in the individual monograph.

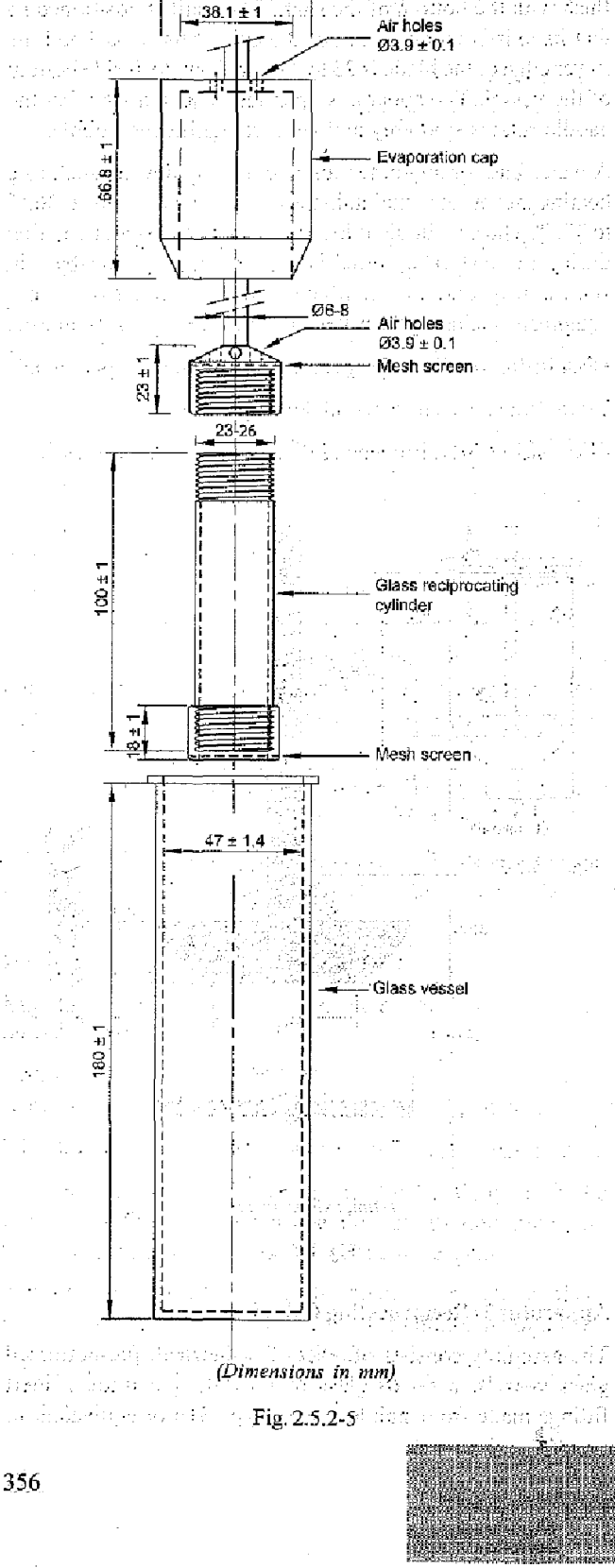


Fig. 2.5.2-5

Dissolution medium. Proceed as directed under Apparatus 1.

Time. Proceed as directed under Apparatus 1.

Filter suitability. Proceed as directed under Apparatus 1.

Apparatus 4 (Flow-Through Cell)

The assembly consists of a reservoir and a pump for the dissolution medium, a flow-through cell and a water bath set to maintain the dissolution medium at 36.5° to 37.5°. Use the specified cell type as given in the individual monograph. The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range between 240 and 960 ml per hour with standard flow rates of 4, 8 and 16 ml per minute. It must deliver a constant flow which is ± 5 per cent of the nominal flow rate, the flow profile is sinusoidal with a pulsation of 110 to 130 pulses per minute. A pump without pulsation may also be used. Dissolution test procedures using a flow-through cell must be characterized with respect to rate and any pulsation. The flow-through cell (see Fig. 2.5.2-6 and Fig. 2.5.2-7) of transparent and inert material is mounted vertically with a filter system specified in the individual monograph that prevents escape of undissolved particles from the top of the cell, standard cell diameters are 12 and 22.6 mm, the bottom cone is usually filled with small glass beads of about 1mm diameter and with one bead of about 5 mm positioned at the apex to protect the fluid entry tube and a tablet holder (see Fig. 2.5.2-6 and Fig. 2.5.2-7) is available for positioning of special dosage forms, for example, inlay tablets. The cell is immersed in a water-bath and the temperature is maintained at 36.5° to 37.5°.

An apparatus that permits observation of the preparation under examination inside the flow-through cell during the test is preferable.

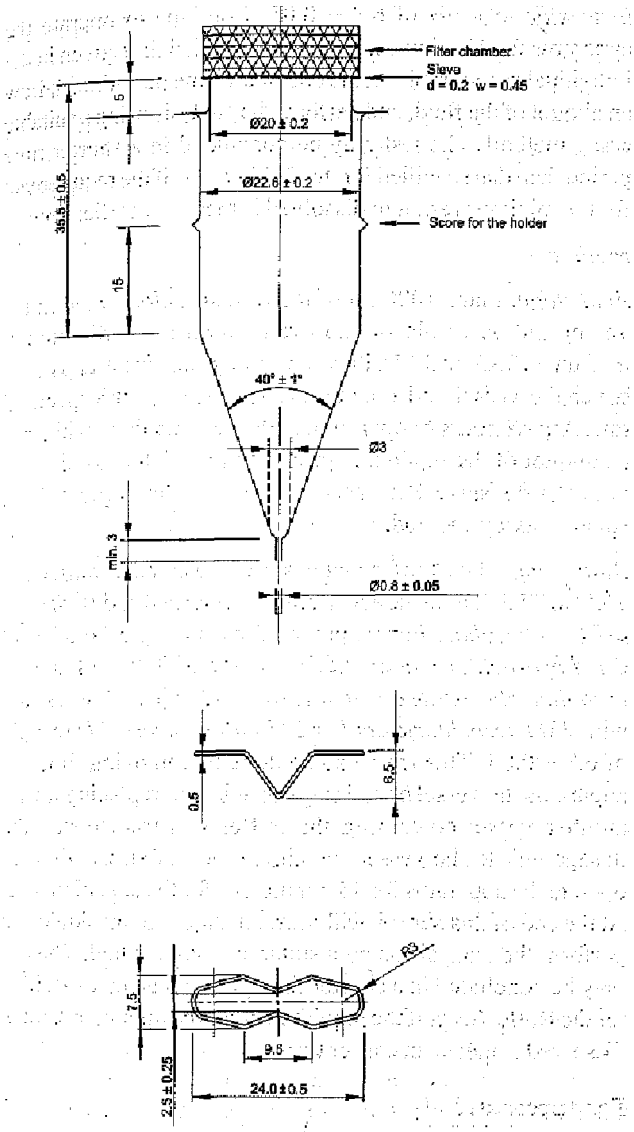


Fig. 2.5.2-6 : Large cell for tablet, capsule and suspension (top), tablet holder for the large cell (bottom).

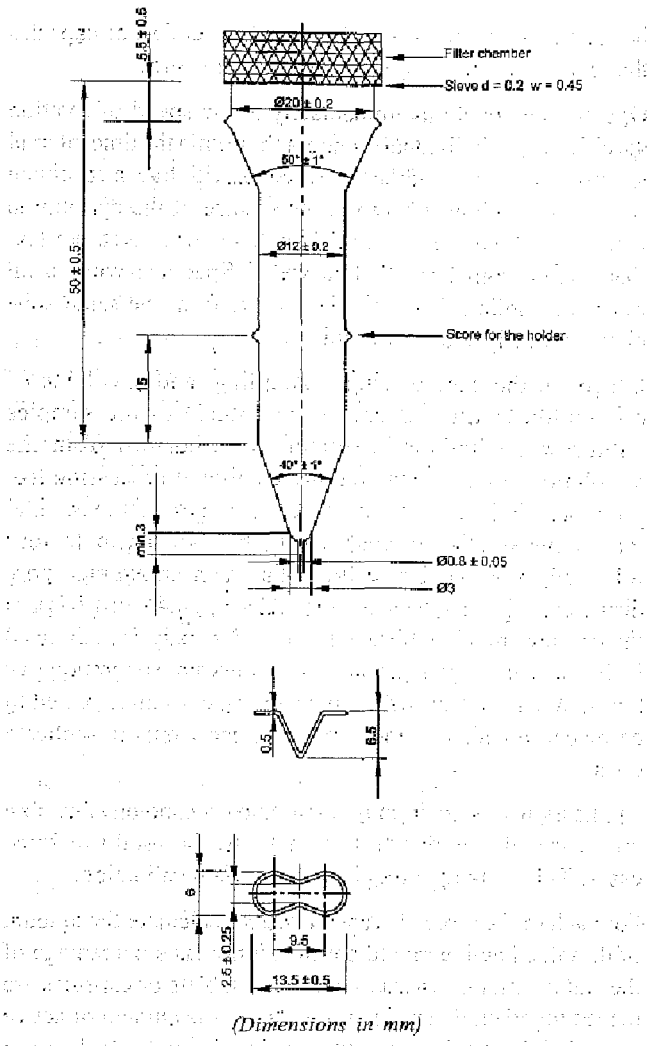


Fig. 2.5.2-7: Small cell for tablet, capsule and suspension (top), tablet holder for the small cell (bottom)

The apparatus uses a clamp mechanism and two O-rings to assemble the cell. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about 1.6 mm inner diameter and chemically inert flanged-end connections.

Dissolution medium. Proceed as directed under Apparatus 1.

Time. Proceed as directed under Apparatus 1.

Filter suitability. Proceed as directed under Apparatus 1.

Methods

For Apparatus 1 and Apparatus 2

Conventional and prolonged-release solid dosage forms

Place the stated volume of the dissolution medium, free from dissolved air, into the vessel of the apparatus. Assemble the apparatus and warm the dissolution medium to 36.5° to 37.5°. Unless otherwise stated, introduce individual dosage unit simultaneously and in a reproducible way in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit. When Apparatus 1 is used, place the tablet or capsule in a dry basket at the beginning of each test. Lower the basket into position before rotation. When Apparatus 2 is used, allow the tablet or capsule to sink to the bottom of the vessel prior to the rotation of the paddle. A suitable device such as a sinker made up of stainless steel, type 316 or any other inert material may be used to keep the dosage unit

horizontal at the bottom of the vessel for tablets or capsules that would otherwise float or stick to the vessel.

Operate the apparatus immediately at the speed of rotation specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the dissolution medium and the top of the rotating blade or basket, not less than 10 mm from the wall of the vessel. Specimen withdrawal at each sampling time point should be from the same zone either manually or automatically.

Except in the case of single sampling, add a volume of dissolution medium equal to the volume of the samples withdrawn. After the specimen is withdrawn from the dissolution vessel, it may require additional processing like filtration, dilution, stirring and special storage conditions. Filter the sample solution through a frit filter (10 µm to 15 µm) followed by a membrane filter disc with an average pore diameter not greater than 1.0 µm (0.22 µm to 0.45 µm). Discard the first few ml of the filtrate. Perform the analysis as directed in the individual monograph. Repeat the whole operation five times. Where two or more tablets or capsules are directed to be placed together in the apparatus, carry out six replicate tests.

Application of direct in situ-measurement technology like fibre optics into dissolution testing may also be useful in some cases. This technique requires appropriate validation.

For each of the tablet or capsule tested, calculate the amount of dissolved active ingredient in solution as a percentage of the stated amount where two or more tablets or capsules are placed together, determine for each test the amount of active ingredient in solution per tablet or capsules and calculate as a percentage of the stated amount.

Modified-release dosage forms

Gastro-resistant dosage forms. Use method A or Method B.

Method A

Acid stage. Place 750 ml of *0.1M hydrochloric acid* in the vessel, and assemble the apparatus. Warm the dissolution medium to 36.5° to 37.5°. Place one dosage unit in the apparatus, cover the vessel and operate the apparatus at the specified rate. After 2 hours of operation in the acid medium, withdraw an aliquot of the liquid and proceed immediately as directed under Buffer stage. Perform the analysis of the aliquot using a suitable assay method.

Buffer stage. Complete the operations of adding the buffer and adjusting the pH within 5 minutes. With the apparatus operating at the rate specified, add to the medium in the vessel 250 ml of a 0.2M solution of *trisodium phosphate dodecahydrate* that has been warmed to 36.5° to 37.5°. Adjust, if necessary, with *2M hydrochloric acid* or *2M sodium hydroxide* to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 minutes, or for the specified time given in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for the minimum amount dissolved is met at an earlier time.

Method B

Acid stage. Place 1000 ml of *0.1M hydrochloric acid* in the vessel and assemble the apparatus. Warm the dissolution medium to 36.5° to 37.5°. Place one dosage unit in the apparatus, cover the vessel and operate the apparatus at the specified rate. After 2 hours of operation in the acid medium, withdraw an aliquot of the liquid and proceed immediately as directed under Buffer stage. Perform the analysis of the aliquot using a suitable assay method.

Buffer stage. Use buffer that has previously been warmed to 36.5° to 37.5°. Drain the acid from the vessel and add 1000 ml of *pH 6.8 phosphate buffer*, prepared by mixing 3 volumes of *0.1M hydrochloric acid* with 1 volume of 0.2M solution of *trisodium phosphate dodecahydrate* and adjust, if necessary, with *2M hydrochloric acid* or *2M sodium hydroxide* to a pH of 6.8 ± 0.05. This may also be done by removing from the apparatus the vessel containing the acid and replacing it with another vessel containing the buffer and transferring the dosage unit to the vessel containing the buffer. Continue to operate the apparatus for 45 minutes, or for the specified time. At the end of this period, withdraw an aliquot of the liquid and perform the analysis using a suitable assay method. The test may be concluded in a shorter time period than that specified for the Buffer Stage if the requirement for the minimum amount dissolved is met at an earlier time.

For Apparatus 3

Conventional and prolonged- release dosage forms

Place the stated volume of the dissolution medium in each vessel of the apparatus. Assemble the apparatus, and warm the dissolution medium to 36.5° to 37.5°. Place one dosage unit in each of the six reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified in the individual monograph. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9 to 10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the solution under test from a zone midway between the surface of the dissolution medium and the bottom of each vessel.

Modified-release dosage forms

Gastro-resistant dosage forms. Use method A or Method B.

Proceed as directed for modified-release dosage forms, Method B under Apparatus 1 and Apparatus 2 using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media and using the volume of medium specified usually 250 ml unless otherwise specified in the individual monograph.

For Apparatus 4

Conventional and prolonged- release dosage forms

Place the glass beads into the cell specified in the monograph. Place one dosage unit on top of the beads or if specified in the monograph on a dosage holder. Assemble the filter head, and fix the parts together by means of a suitable clamping device. Introduce by the pump the dissolution medium warmed to 36.5° to 37.5° through the bottom of the cell to obtain the flow rate specified in the individual monograph and measured with an accuracy of 5 per cent. Collect the eluate by fractions at each of the times stated. Apparatus 4 can be operated in open loop and closed loop mode.

Perform the analysis as directed in the individual monograph. Repeat the test with additional dosage units.

Modified-release dosage forms

Gastro-resistant dosage forms. Use method A or Method B.

Proceed as directed for modified-release dosage forms under Apparatus 1 and Apparatus 2, using the specified media.

Acceptance criteria for different dosage forms

Conventional-release dosage forms

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units conform to Table 1. If the results do not conform to the requirements at stage S_1 given in the table, continue testing with additional dosage units through stages S_2 and S_3 unless the results conform at stage S_2.

Where capsule shells interfere with the analysis, remove the contents of not less than 6 capsules as completely as possible, and dissolve the empty capsule shells in the specified volume of the dissolution medium. Perform the analysis as directed in the individual monograph. Make any necessary correction.

Correction factors should not be greater than 25 per cent of the stated amount.

Table 1

Level	Number tested	Acceptance criteria
S_1	6	Each unit is not less than Q* + 5 per cent**.
S_2	6	Average of 12 units (S_1 + S_2) is equal to or greater than Q, and no unit is less than Q − 15 per cent**.
S_3	12	Average of 24 units (S_1+S_2+S_3) is equal to or greater than Q, not more than 2 units are less than Q − 15 per cent** and no unit is less than Q − 25 per cent**.

*Q is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labelled content.

**Percentages of the labelled content.

Prolonged-release dosage forms

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units conform to Table 2. If the results do not conform to the requirements at stage L_1 given in the table, continue testing with additional dosage units through stages L_2 and L_3 unless the results conform at stage L_2.

The limits embrace each value of Q, the amount dissolved at each specified dosing interval. Where more than one range is specified, the acceptance criteria apply to each range.

Table 2

Level	Number tested	Acceptance criteria
L_1	6	No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time.
L_2	6	The average value of the 12 units (L_1 + L_2) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10 per cent of labelled content outside each of the stated ranges; and none is more than 10 per cent of labelled amount below the stated amount at the final test time.
L_3	12	The average value of the 24 units (L_1 + L_2 + L_3) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10 per cent of labelled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10 per cent of labelled content below the stated amount at the final test time; and none of the units is more than 20 per cent of labelled content outside each of the stated ranges or more than 20 per cent of labelled content below the stated amount at the final test time.

Modified- release dosage forms

Acid stage. Unless otherwise specified, the requirements of this part of the test are met if the quantities, based on the

percentage of the labelled content of active substance dissolved from the units tested conform to Table 3. Continue the testing through the 3 levels unless the results of both acid and buffer stages conform at an earlier level.

Table 3

Level	Number tested	Acceptance criteria
A_1	6	No individual value exceeds 10 per cent dissolved.
A_2	6	The average value of the 12 units ($A_1 + A_2$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.
A_3	12	The average value of the 24 units ($A_1 + A_2 + A_3$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.

Buffer stage. Unless otherwise specified, the requirements of this part of the test are met if the quantities, based on the percentage of the labelled content of active substance dissolved from the units tested conform to Table 4. Continue the testing through the 3 levels unless the results of both acid and buffer stages conform at an earlier level. The value of Q in Table 4 is 75 per cent dissolved unless otherwise specified. The quantity, Q, is the specified total amount of active substance dissolved in both the acid and buffer stages, expressed as a percentage of the labelled content.

Table 4

Level	Number tested	Acceptance criteria
B_1	6	No unit is less than Q + 5 per cent*
B_2	6	The average value of the 12 units ($B_1 + B_2$) is equal to or greater than Q,and no unit is less than Q – 15 per cent*.
B_3	12	The average value of 24 units ($B_1 + B_2 + B_3$) is equal to or greater than Q, not more than 2 units are less than Q – 15 per cent*, and no unit is less than Q – 25 per cent*.

* *percentages of the labelled content.*

Apparatus suitability

The determination of suitability of a test assembly to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as stated. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotation speed (Apparatus 1 and Apparatus 2), dip rate (Apparatus 3) and flow rate of medium (Apparatus 4).

Performance verification test, Apparatus 1 and Apparatus 2. The suitability for the individual apparatus is demonstrated by the performance verification test using *prednisone tablet IPRS* periodically, according to the operating conditions specified in the technical data sheet. The apparatus is suitable if the results obtained are within the acceptance range stated in the technical data sheet specific to the lot of *prednisone tablet IPRS* used.

2.5.3. Uniformity of Weight of Single-Dose Preparations

Weigh individually 20 units selected at random or, for single-dose preparations in individual containers, the contents of 20 units, and calculate the average weight. Not more than two of the individual weights deviate from the average weight by more than the percentage shown in the table and none deviates by more than twice that percentage.

Table

Dosage form	Average weight	Percentage deviation
Uncoated and film-coated tablets	80 mg or less	10
	More than 80 mg but less than 250 mg	7.5
	250 mg or more	5
Capsules, granules and powders (single-dose)	Less than 300 mg	10
	300 mg or more	7.5
Powders for parenteral use*	More than 40 mg	10
Pessaries and suppositories	All weights	5

*When the average weight is equal to or below 40 mg, the preparation is not submitted to the test for uniformity of weight but to the test for uniformity of content of single-dose preparations.

For capsules. Weigh an intact capsule. Open it without losing any part of the shell and remove the contents as completely as possible. For soft gelatin capsules, wash the shell with a suitable solvent and keep aside until the odour of the solvent is not perceptible. Weigh the shell. The difference between the weighings gives the weight of the contents. Repeat the procedure with another 19 capsules.

For powders for parenteral use. Remove any adhering labels from a container and wash and dry the outside. Open the container and immediately weigh it along with the contents.

Empty it as completely as possible by gentle tapping, rinse it with *water* and then with *ethanol*. Dry at 100° to 105° for one hour, or if the nature of the contents does not permit drying at this temperature, dry at a lower temperature to constant weight. Cool in a desiccator and weigh. The difference in the weighings gives the weight of the contents of the container. Repeat the procedure with another 19 containers.

2.5.4. Uniformity of Content of Single-Dose Preparations

The test for uniformity of content of single-dose preparations is based on the assay of the individual contents of active substance(s) of a number of single-dose units to determine whether the individual contents are within limits set with reference to the average content of the sample.

Method. Determine the content of active ingredient(s) in each of 10 dosage units taken at random using the method given in the monograph or by any other suitable analytical method.

Acceptance limits

For tablets, powders and suspensions for injection and ophthalmic inserts:

The preparation complies with the test if each individual content is 85 to 115 per cent of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75 to 125 per cent of the average content.

If one individual content is outside the limits of 85 to 115 per cent of the average content but within the limits of 75 to 125 per cent, repeat the determination using another 20 dosage units. The preparation complies with the test if not more than one of the individual contents of the total sample of 30 dosage units is outside 85 to 115 per cent of the average content and none is outside the limits of 75 to 125 per cent of the average content.

For capsules, powders other than for parenteral use, granules, pessaries and suppositories:

The preparation complies with the test if not more than one individual content is outside the limits of 85 to 115 per cent of the average content and none is outside the limits of 75 to 125 per cent of the average content. The preparation fails to comply with the test if more than three individual contents are outside the limits of 85 to 115 per cent of the average content or if one or more individual contents are outside the limits of 75 to 125 per cent of the average content.

If two or three individual contents are outside the limits of 85 to 115 per cent of the average content but within the limits of 75 to 125 per cent, repeat the determination using another 20 dosage units. The preparation complies with the test if not more than three individual contents of the total sample of 30 dosage units are outside the limits of 85 to 115 per cent of the average content and none is outside the limits of 75 to 125 per cent of the average content.

2.5.4. (i) Uniformity of Dosage Units

To ensure the consistency of dosage units, each unit in a batch should have active ingredient/substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of an active substance in each dosage unit. Unless otherwise stated, the uniformity of dosage units specification is not intended to apply to solutions, suspensions, emulsions or gels in single-dose containers intended for local action following cutaneous administration. The test for content uniformity is not required for multivitamin, single-vitamin and trace-element preparations.

The term 'uniformity of dosage unit' is defined as the degree of uniformity in the amount of the active ingredient among dosage units. Therefore, the requirements of this chapter apply to each active ingredient being comprised in dosage units containing one or more active ingredient(s), unless otherwise specified elsewhere in this Pharmacopoeia.

The uniformity of dosage units can be demonstrated by either of two methods: uniformity of content or weight variation (see Table 1).

The test for uniformity of content of preparations presented in dosage units is based on the assay of the individual contents of active ingredient(s) in a number of dosage units to determine whether the individual contents are within the limits set. The uniformity of content method may be applied in all cases.

The test for weight variation is applicable for the following dosage forms:

(1) Solutions enclosed in single-dose containers and in soft capsules;

(2) Solids (including powders, granules and sterile solids) that are packaged in single-dose containers and contain no active or inactive added substances;

(3) Solids (including sterile solids) that are packaged in single-dose containers, with or without active or inactive added substances, that have been prepared from true solutions and freeze-dried in the final containers and are labelled to indicate this method of preparation;

(4) Hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of an active ingredient comprising 25 per cent or more, by weight, of the dosage unit or, in the case of hard capsules, the capsule contents,

except that uniformity of other active substances present in lesser proportions is demonstrated by meeting uniformity of content requirements.

The test for uniformity of content is required for all dosage forms not meeting the above conditions for the weight variation test.

Table 1 – Application of Uniformity of Content (CU) and Weight Variation (WV) test for dosage forms

Dosage Forms	Type	Sub-type	Dose and ratio of active substance	
			≥ 25 mg and ≥ 25 per cent	< 25 mg or < 25 per cent
Tablets	uncoated		WV	CU
	coated	film	WV	CU
		others	CU	CU
Capsules	hard		WV	CU
	soft	suspensions, emulsions, gels	CU	CU
		solutions	WV	WV
Solids in single-dose containers	single component		WV	WV
	multiple components	solution dried freeze-in final container	WV	WV
		others	CU	CU
Solutions enclosed in single-dose containers and into soft capsules			WV	WV
Others			CU	CU

Uniformity of content

Select 30 units, and proceed as follows for the dosage form designated. Where different procedures are used for assay of the preparation and for the uniformity of content test, it may be necessary to establish a correction factor to be applied to the results of the latter.

Solid dosage forms. Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see Table 2).

Liquid or semi-solid dosage forms. Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use and express the results as delivered dose. Calculate the acceptance value (see Table 2).

NOTE — In the chapter, unit and dosage unit are synonymous.

Calculation of Acceptance Value

Calculate the Acceptance Value (*AV*) using the following formula for which the terms are as defined in Table 2.

$$\left|M - \overline{X}\right| + ks$$

Weight variation

Carry out an assay for the active ingredient(s) on a representative sample of the batch using an appropriate analytical method. This value is result *A*, expressed as percentage of label claim (see Calculation of Acceptance Value). Assume that the concentration (weight of active substance per weight of dosage unit) is uniform. Select not less than 30 dosage units, and proceed as follows for the dosage form designated.

Uncoated or film-coated tablets. Weigh individually 10 tablets, calculate the active ingredient content expressed as percentage of label claim, of each tablet from the weight of the individual tablets and the result of the assay. Calculate the acceptance value.

Hard capsules. Weigh intact 10 capsules individually, open it without losing any part of the shell and remove the contents as completely as possible. Weigh the shell individually. The difference between the weighing gives the weight of the contents. Calculate the active substance content of each capsule from the weight of content removed from the individual capsules and the result of the assay. Calculate the acceptance value.

Soft capsules. Weigh accurately 10 intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the active substance content in each capsule from the weight of product removed from the individual capsules and the result of the assay. Calculate the acceptance value.

Solid dosage forms other than tablets and capsules. Proceed as directed for hard capsules, treating each unit as described therein. Calculate the acceptance value.

Liquid or semi-solid dosage forms. Weigh accurately the amount of liquid or semi-solid that is removed from each of 10

Table 2

Variable	Definition	Conditions	Value
$\overline{X}$	Mean of individual contents ($x_1, x_2, ..., x_n$), expressed as a percentage of the label claim		
$x_1, x_2, ..., x_n$	Individual contents of the dosage units tested, expressed as a percentage of the label claim		
n	Sample size (number of dosage units in a sample)		
k	Acceptability constant	If $n = 10$, then k =	2.4
		If $n = 30$, then k =	2.0
s	Sample standard deviation		$\left[\frac{\sum_{i=1}^{n}(x_i - \overline{X})^2}{n-1}\right]^{1/2}$
RSD	Relative standard deviation		$\frac{100s}{\overline{X}}$
M (case 1) to be applied when $T \leq 101.5$	Reference value	If 98.5 per cent $\leq \overline{X} \leq$ 101.5 per cent, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X}$ < 98.5 per cent, then	M = 98.5 per cent ($AV = 98.5 - \overline{X} + ks$)
		If $\overline{X}$ > 101.5 per cent, then	M = 101.5 per cent ($AV = \overline{X} - 101.5 + ks$)
M (case 2) to be applied when $T > 101.5$	Reference value	If 98.5 per cent $\leq \overline{X} \leq T$, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X}$ < 98.5 per cent, then	M = 98.5 per cent ($AV = 98.5 - \overline{X} + ks$)
		If $\overline{X} > T$, then	$M = T$ per cent ($AV = \overline{X} - T + ks$)
Acceptance value (AV)			General formula: $\left\lvert M - \overline{X} \right\rvert + ks$ (Calculations are specified above for the different cases)
$L1$	Maximum allowed acceptance value		$L1$ = 15.0 unless otherwise specified

Contd.

Variable	Definition	Conditions	Value
L2	Maximum allowed range for deviationof each dosage unit tested from the calculated value of *M*	On the low side, no dosage unit result can be less than (1–(0.01) (*L2*)) *M* while on the high side, no dosage unit result can be more than (1 + (0.01) (*L2*)) *M* (This is based on an *L2* value of 25.0)	*L2*= 25.0 unless otherwise specified
T	Target content per dosage unit at time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, *T* is equal to 100 per cent or *T* is the manufacturer's approved target content per dosage unit		

individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the active ingredient content in each container from the weight of product removed from the individual containers and the result of the assay. Calculate the acceptance value.

Calculation of Acceptance value

Calculate the acceptance value (*AV*) as shown in uniformity of content, except that the individual contents of the units are replaced with the individual estimated contents defined below.

$x_1, x_2, \ldots, x_n$ = individual estimated contents of the dosage units tested, using the formula:

$$x_i = w_i \times \frac{A}{\overline{W}}$$

where,

$w_1, w_2, \ldots, w_n$ = individual weights of the dosage units tested;

A = content of active substance (percentage of label claim) obtained using an appropriate analytical method (assay);

$\overline{W}$ = mean of individual weights ($w_1, w_2, \ldots, w_n$).

Criteria. Unless otherwise specified, apply the following criteria.

Solid, semi-solid and liquid dosage forms. The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to *L1* per cent. If the acceptance value is more than *L1* per cent, test the next 20 dosage units and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to *L1* per cent and no individual content of the any dosage unit is less than (1–(0.01) (*L2*)) *M* nor more than (1 + (0.01) (*L2*)) *M* as specified in the calculation of acceptance value under uniformity of content or under weight variation. Unless otherwise specified, *L1* is 15.0 and *L2* is 25.0.

2.5.5. Friability of Uncoated Tablets

This test is applicable to compressed tablets and is intended to determine the physical strength of tablets.

Apparatus. It consists of a drum of transparent synthetic polymer with polished internal surfaces and subject to minimum static build-up. It has a diameter of 283-291 mm and a depth of 36-40 mm (Fig. 2.5.5); one side of the drum is removable. A curved projection with an inner radius of 75.5 mm to 85.5 mm and extending from the middle of the drum to the outer wall enables the tumbling of the tablets at each turn of the drum. The outer diameter of the central ring is 24.5 mm to 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 rpm. It should be ensured that with every turn of the drum the tablets roll or slide and fall onto the drum wall or onto each other.

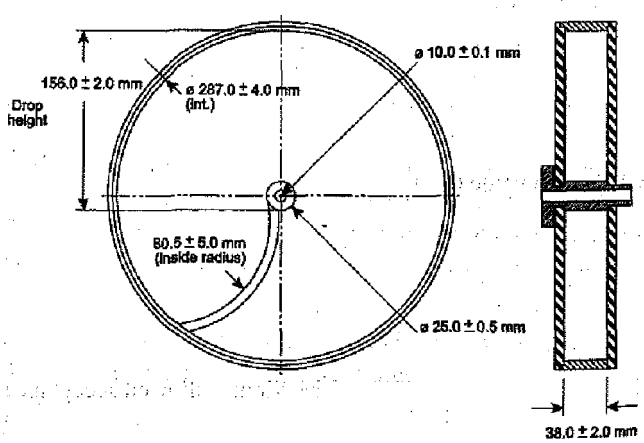


Fig. 2.5.5: Tablet friability apparatus

Method. For tablets with an average weight of 0.65 g or less take a sample of whole tablets corresponding to about 6.5 g and for tablets with an average weight of more than 0.65 g take a sample of 10 whole tablets.

Dedust the tablets carefully and weigh accurately the required number of tablets. Place the tablets in the drum and rotate it 100 times. Remove the tablets, remove any loose dust from them and weigh them accurately. The test is run only once unless the results are difficult to interpret or if the weight loss is greater than the targeted value, in which case, the test is repeated twice and the mean of the three tests is determined. A maximum loss of weight (from a single test or from the mean of the three tests) not greater than 1.0 per cent is acceptable for most tablets.

If obviously cracked, chipped or broken tablets are present in the sample after tumbling, the sample fails the test.

If the size or shape of the tablet causes irregular tumbling, adjust the drum base so that it forms an angle of about 10° with the horizontal and the tablets do not bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, a humidity controlled environment is required for testing.

A drum with dual scooping projections, or an apparatus with more than one drum, for the running of multiple samples at same time, are also permitted.

2.5.6. Contents of Packaged Dosage Forms

This chapter provides assurance that the amount of material filled or packed into the product or oral liquids when transferred from the original container conforms to the labelled amount. The following tests and specifications apply to oral dosage forms and preparations intended for topical use that are packaged in containers in which the labelled net quantity is not more than 100 g or 300 ml or 1000 units, as the case may be. For higher labelled quantities the test and limits given in the standards of The Legal Metrology (Packaged Commodities) Rule, 2011 may be followed.

Ointments, Creams, Pastes, Gels, Lotions and Powders

For Containers labelled by Weight

Select a sample of 10 filled containers and remove any labelling that might be altered in weight while removing the contents of the containers. Clean and dry the outer surfaces of the containers and weigh each container. Remove quantitatively the contents from each container. If necessary, cut open the container and wash each empty container with a suitable solvent, taking care to ensure that the closure and other parts of the container are retained. Dry and again weigh each empty container together with its parts which may have been removed. The difference between the two weights is the net weight of the contents of the container.

For Containers labelled by Volume

Select a sample of 10 filled containers and determine the weight of the contents of each container as directed above for Containers labelled by Weight. Determine the weight per ml (2.4.29) and calculate the net volume of the contents of each container.

Alternatively, pour completely the contents of each container into calibrated volume measures of the appropriate size and determine the volume of the contents of each of the 10 containers. Other methods of determining the density may be employed depending on the formulation. Similarly, if the container contents are less than 25 ml, smaller calibrated volume measures may be used, with the quantities of miscible liquid adjusted accordingly.

Acceptance Criteria

The average net weight of the contents of the 10 containers is not less than the labelled amount and the net weight of the contents of any single container is not less than 90 per cent of the labelled amount where the labelled amount is 60 g or 60 ml or less, or not less than 95 per cent of the labelled amount where the labelled amount is more than 60 g or 60 ml.

If this requirement is not met, determine the net weight of the contents of 20 additional containers. The average net weight of the contents of the 30 containers is not less than the labelled amount, and the net weight of the contents of not more than 1 of the 30 containers is less than 90 per cent of the labelled amount where the labelled amount is 60 g or 60 ml or less, or less than 95 per cent of the labelled amount is more than 60 g or 60 ml. None of individual weights deviates by more than twice that of respective minimum percentage limit.

Liquids, Suspensions, Granules and Powders for oral liquids

Select not less than 30 containers for the determination of net volume and proceed as follows for the dosage form designated.

Oral Solution and Oral Suspension

NOTE — Allow drainage for not more than 5 seconds for single unit containers and not more than 10 minutes for multiple unit containers, unless otherwise specified in the monograph.

Select and shake a sample of 10 containers individually. Pour completely the contents of each container into a tared container of the appropriate size. Determine the weight of the contents and calculate the volume using the density.

Constituted Solution from Powders and Suspensions. Select and constitute a sample of 10 containers with the volume of diluent stated in the labelling, accurately measured and shake

individually. Determine the weight of the contents of each container as directed under Oral Solution and Oral Suspension.

Alternatively, the following by volume procedure may be used, as instructed in the labelling, carefully discharge the contents of each container into separate dry graduated cylinders of a rated capacity not exceeding 2.5 times the volume to be measured and calibrated (See 2.1.6. Volumetric Glassware). Care must be taken to avoid the formation of air bubbles during the process. In the absence of labelling instructions, support the containers at about 30° angle to the horizontal and gently discharge the contents into the graduated cylinder.

Determine the volume of each mixture, when free from bubbles.

Acceptance Criteria

For multiple unit containers (See Fig. 2.5.6-1) —

The average net volume of the contents of the 10 containers is not less than the labelled amount, and the net volume of the contents of any single container is not less than 95 per cent of the labelled amount.

If this requirement is not met and (A), the average net volume of the contents is less than the labelled amount, and the net volume of the contents of any single container is not less than 95 per cent of the labelled amount, or if (B), the average net volume of the contents is not less than the labelled amount, and the net volume of the contents of the any single container

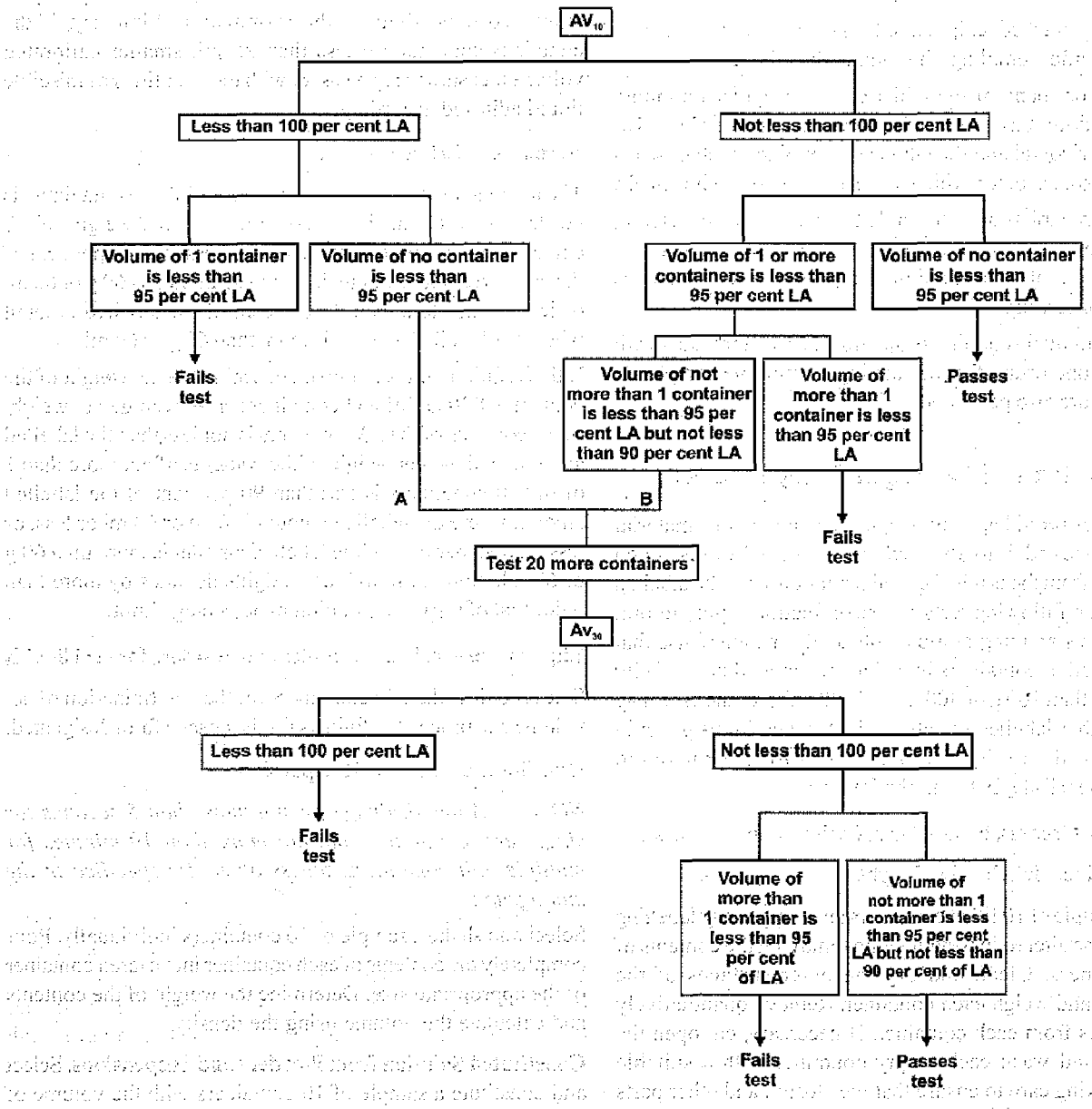

AV_{10}
Less than 100 per cent LA
Not less than 100 per cent LA
Volume of 1 container is less than 95 per cent LA
Volume of no container is less than 95 per cent LA
Volume of 1 or more containers is less than 95 per cent LA
Volume of no container is less than 95 per cent LA
Fails test
Volume of not more than 1 container is less than 95 per cent LA but not less than 90 per cent LA
Volume of more than 1 container is less than 95 per cent LA
Passes test
A
B
Fails test
Test 20 more containers
Av_{30}
Less than 100 per cent LA
Not less than 100 per cent LA
Fails test
Volume of more than 1 container is less than 95 per cent of LA
Volume of not more than 1 container is less than 95 per cent LA but not less than 90 per cent of LA
Fails test
Passes test

Fig. 2.5.6-1: Decision flow diagram for multiple unit containers (AV = average volume; LA = labelled amount)

is less than 95 per cent, but is not less than 90 per cent of the labelled amount, determine the net volume of the contents of 20 additional containers.

The average net volume of the contents of the 30 containers is not less than the labelled amount, and the net volume of the contents of not more than 1 of the 30 containers is less than 95 per cent of the labelled amount, but not less than 90 per cent of the labelled amount.

Acceptance Criteria

For single unit containers (See Fig. 2.5.6-2) —

The average net volume of the contents of the 10 containers is not less than the labelled amount, and the net volume of the contents of each of the 10 containers is not less than 95 per cent and not more than 110 per cent of the labelled amount.

If this requirement is not met and (A), the average net volume of the contents is less than the labelled amount, and the net volume of the contents of any single container is not outside the range of 95 per cent to 110 per cent of the labelled amount, or if, (B), the average net volume of the contents is not less than the labelled amount, and the net volume of the contents of not more than 1 container is outside the range of 95 per cent

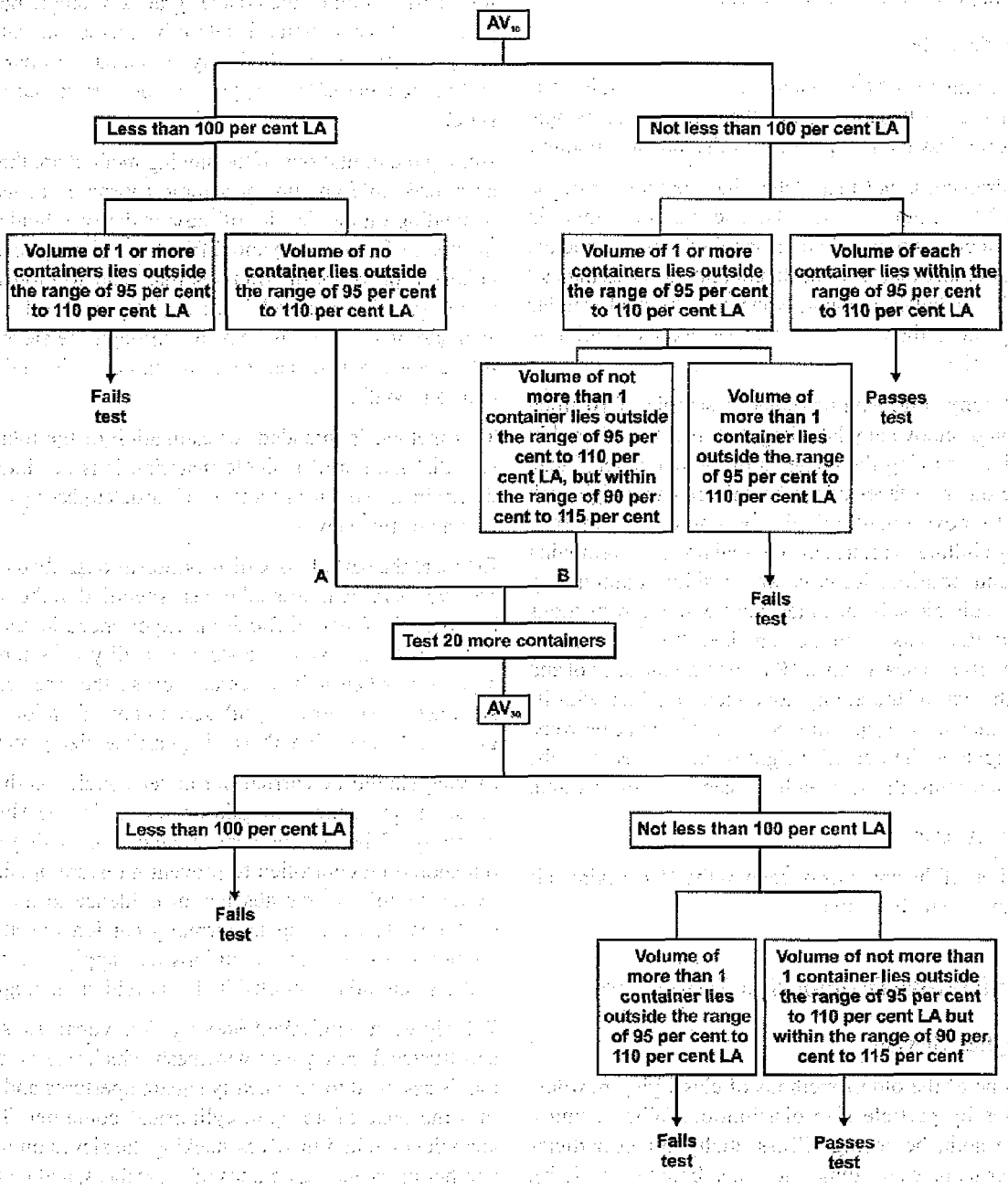

AV_{10}
Less than 100 per cent LA
Not less than 100 per cent LA
Volume of 1 or more containers lies outside the range of 95 per cent to 110 per cent LA
Volume of no container lies outside the range of 95 per cent to 110 per cent LA
Volume of 1 or more containers lies outside the range of 95 per cent to 110 per cent LA
Volume of each container lies within the range of 95 per cent to 110 per cent LA
Fails test
Volume of not more than 1 container lies outside the range of 95 per cent to 110 per cent LA, but within the range of 90 per cent to 115 per cent
Volume of more than 1 container lies outside the range of 95 per cent to 110 per cent LA
Passes test
A
B
Fails test
Test 20 more containers
AV_{30}
Less than 100 per cent LA
Not less than 100 per cent LA
Fails test
Volume of more than 1 container lies outside the range of 95 per cent to 110 per cent LA
Volume of not more than 1 container lies outside the range of 95 per cent to 110 per cent LA but within the range of 90 per cent to 115 per cent
Fails test
Passes test

Fig. 2.5.6-2: Decision flow diagram for single unit containers (AV = average volume; LA = labelled amount)

to 110 per cent of the labelled amount, but within the range of 90 per cent to 115 per cent of the labelled amount, determine the net volume of the contents of 20 additional containers.

The average net volume of the contents of the 30 containers is not less than the labelled amount, and the net volume of the contents of not more than 1 of the 30 containers is outside 95 per cent to 110 per cent of the labelled amount, but within the range of 90 per cent to 115 per cent of the labelled amount.

Capsules, Pessaries , Suppositories and Tablets. Select a sample of 10 containers and determine the number of capsules, pessaries, suppositories or tablets in each container.

Acceptance Criteria

The average number of the contents in the 10 containers is not less than the labelled amount and the number in any single container is not less than 98 per cent of the labelled amount.

If this requirement is not met, determine the number of the contents in 20 additional containers. The average number in the 30 containers is not less than the labelled amount, and the number in not more than 1 of the 30 containers is less than 98 per cent of the labelled amount. None of individual weights deviates by more than twice that of respective minimum percentage limits.

Aerosols, Sprays and Foams. Select a sample of 10 filled containers and remove any labelling that might be altered in weight while removing the contents of the container. Clean and dry the outer surfaces of the containers and weigh each container. Remove quantitatively the contents from each container by chilling to reduce the internal pressure, removing the valve and pouring. Remove any residual contents by rinsing with suitable solvents, then rinse with a few portions of *methanol*. Retain as a unit the container, the valve and all associated parts and heat them at 100° for 5 minutes. Cool and again weigh each of the empty containers together with its parts which may have been removed. The difference between the two weights is the net fill weight of the contents of the container. Determine the net weight for each container tested.

Acceptance Criteria

The net weight of the contents of each of the 10 containers is not less than the labelled amount.

2.5.7. Particle Size Distribution Estimation

A. By Analytical Sieving

Sieving is one of the oldest methods of classifying powders and granules by particle size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e., breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 µm. For smaller particles, the light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials, other means of agitation such as air-jet sieving or sonic sifting may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75 µm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of test sieves) and difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on one or two sieves.

Estimate the particle size distribution as described under *Dry Sieving Method*, unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e., material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 µm), serious consideration should be given to the use of an alternative particle-sizing method.

Sieving should be carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out should be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material should be detailed in the individual monograph.

Principles of Analytical Sieving. Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is sealed into the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve.

The nest of sieves is subjected to a standardized period of agitation, and then the weight of material retained on each sieve is accurately determined. The test gives the weight percentage of powder in each sieve size range.

This sieving process for estimating the particle size distribution of a single pharmaceutical powder is generally intended for use where at least 80 per cent of the particles are larger than 75 µm. The size parameter involved in determining particle size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

Test Sieves

Test sieves suitable for pharmacopeial tests conform to the most current edition of International Organization for Standardization Specification ISO 3310-1: Test Sieves - Technical Requirements and Testing (see Table 1). Unless otherwise specified in the monograph, use those ISO sieves listed as principal sizes in Table 1. Unless otherwise specified in the monograph, use those ISO sieves listed in Table 1 as recommended in the particular region.

Table 1 - Sizes of Standard Sieve Series in Range of Interest

ISO Nominal Aperture			US	Recom-	Euro-	Japan-
Principal	Supplementary		Sieve	mended	pean	ese
Sizes	Sizes		No.	Sieve	Sieve	Sieve
R 20/3	R 20	R 40/3		(microns)	No.	No.
11.20 mm	11.20 mm	11.20 mm			11200	
	10.00 mm					
		9.50 mm				
	9.00 mm					
8.00 mm	8.00 mm	8.00 mm				
	7.10 mm					
		6.70 mm				
	6.30 mm					
5.60 mm	5.60 mm	5.60 mm			5600	3.5
	5.00 mm					
		4.75 mm				4
	4.50 mm					
4.00 mm	4.00 mm	4.00 mm	5	4000	4000	4.7
	3.55 mm					
		3.35 mm	6			5.5
	3.15 mm					
2.80 mm	2.80 mm	2.80 mm	7	2800	2800	6.5
	2.50 mm					
		2.36 mm	8			7.5
	2.24 mm					
2.00 mm	2.00 mm	2.00 mm	10	2000	2000	8.6
	1.80 mm					
		1.70 mm	12			10
	1.60 mm					
1.40 mm	1.40 mm	1.40 mm	14	1400	1400	12
	1.25 mm					
		1.18 mm	16			14
	1.12 mm					
1.00 mm	1.00 mm	1.00 mm	18	1000	1000	16
	900 µm					
		850 µm	20			18
	800 µm					
710 µm	710 µm	710 µm	25	710	710	22
	630 µm					
		600 µm	30			26
	560 µm					
500 µm	500 µm	500 µm	35	500	500	30
	450 µm					
		425 µm	40			36
	400 µm					
355 µm	355 µm	355 µm	45	355	355	42
	315 µm					
		300 µm	50			50
	280 µm					
250 µm	250 µm	250 µm	60	250	250	60
	224 µm					
		212 µm	70			70
	200 µm					
180 µm	180 µm	180 µm	80	180	180	83
	160 µm					
		150 µm	100			100
	140 µm					
125 µm	125 µm	125 µm	120	125	125	119
	112 µm					
		106 µm	140			140
	100 µm					
90 µm	90 µm	90 µm	170	90	90	166
	80 µm					
		75 µm	200			200
	71 µm					
63 µm	63 µm	63 µm	230	63	63	235
	56 µm					
		53 µm	270			282
	50 µm					
45 µm	45 µm	45 µm	325	45	45	330
	40 µm					
		38 µm			38	391

Sieves are selected to cover the entire range of particle sizes present in the test sample. A nest of sieves having a $\sqrt{2}$ progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometers or millimeters in denoting test sieve openings.

NOTE—Mesh numbers are provided in the table for conversion purposes only.

Test sieves are made from stainless steel or, less preferably, from brass or other suitable nonreactive wire.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 µm, Standard Glass Spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

Cleaning Test Sieves. Ideally, test sieves should be cleaned using only a low pressure air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

Test sample. If the test sample weight is not given in the monograph for a particular material, use a test sample having a weight between 25 - 100 g, depending on the bulk density of the material, and test sieves having a 200 mm diameter. For 76-mm sieves, the amount of material that can be accommodated is approximately 1/7th that which can be accommodated on a 200-mm sieve. Determine the most appropriate weight for a given material by test sieving accurately weighed samples of different weights, such as 25 g, 50 g, and 100 g, for the same time period on a mechanical shaker.

NOTE—If the test results are similar for the 25g and 50g samples, but the 100g sample shows a lower percentage through the finest sieve, the 100g sample size is too large.

Where only a sample of 10 to 25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g., down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly isodiametrical shape, sample weights below 5 g for a 200 mm screen may be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to absorbing up or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging is not influencing the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 percent (m/m) level to minimize this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

Agitation Methods. Several different sieve and powder agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitude of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), because changes in the agitation conditions will give different results for the sieve analysis and endpoint determinations, and may be sufficiently different to give a failing result under some circumstances.

Endpoint Determination. The test sieving analysis is complete when the weight on any of the test sieves does not change by more than 5 per cent or 0.1 g (10 per cent in the case of 76-mm sieves) of the previous weight on that sieve. If less than 5 per cent of the total sample weight is present on a given sieve, the endpoint for that sieve is increased to a weight change of not more than 20 per cent of the previous weight on that sieve.

If more than 50 per cent of the total sample weight is found on any one sieve, unless this is indicated in the monograph, the test should be repeated, but with the addition to the sieve nest of a more coarse sieve, intermediate between that carrying the excessive weight and the next coarsest sieve in the original nest, i.e., addition of the ISO series sieve omitted from the nest of sieves.

Sieving Methods

Mechanical Agitation

Dry Sieving Method. Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test sample on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 minutes. Then carefully remove each from the nest without loss of material. Reweigh each sieve, and determine the weight of material on each sieve. Determine the weight of material in the collecting pan in a similar manner. Reassemble the nest of sieves, and agitate for 5 minutes. Remove and weigh each sieve as previously described. Repeat these steps

until the endpoint criteria are met (see Endpoint Determination under Test Sieves). Upon completion of the analysis, reconcile the weights of material. Total losses must not exceed 5 per cent of the weight of the original test sample.

Repeat the analysis with a fresh sample, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle size analysis method should be used.

Air Entrainment Methods

Air Jet* and *Sonic Sifter Sieving. Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air jet sieving. It uses the same general sieving methodology as that described under the Dry Sieving Method, but with a standardized air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle size distribution. Air jet sieving often includes the use of finer test sieves than those used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic sifting method, a nest of sieves is used, and the test sample is carried in a vertically oscillating column of air that lifts the sample and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g, when sonic sifting is employed.

The air jet sieving and sonic sieving methods may be useful for powders or granules when mechanical sieving techniques are incapable of giving a meaningful analysis.

These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e., below 75 μm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

Interpretation

The raw data must include the weight of test sample, the total sieving time, and the precise sieving methodology and the set values for any variable parameters, in addition to the weights retained on the individual sieves and in the pan. It may be convenient to convert the raw data into a cumulative weight distribution, and if it is desired to express the distribution in terms of a cumulative weight undersize, the range of sieves used should include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

B) By Laser Light Diffraction

The method is based on the ISO Standards 13320-1 (1999) and 9276-1(1998).

The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider angular range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e., by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For nonspherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g., sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems (e.g., powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids), through analysis of their angular light-scattering patterns. It does not address specific requirements of particle-size measurement of specific products.

Principle

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

Instrument

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity, or direct bright light.

An example of a setup of a laser light diffraction instrument is given in Fig. 2.5.7-1. Other equipment may be used.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size distribution and associated data analysis and reporting.

The particles can enter the laser beam in two positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance. In so-called reversed Fourier optics the particles enter behind the collecting lens and thus in a converging beam. The advantage of the conventional setup is that a reasonable path length for the sample is allowed within the working distance of the lens. The second setup allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

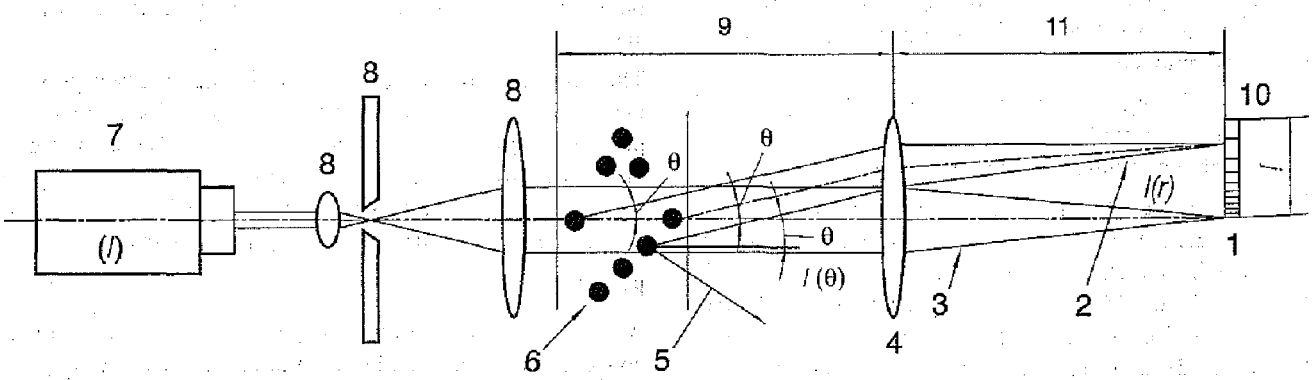
Development of the Method

The measurement of particle size by laser diffraction can give reproducible data, even in the submicron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g., dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately 0.1 µm to 3 mm. Because of recent advances in lens and equipment design, newer instruments are routinely capable of exceeding this range. With the validation report, the user demonstrates the applicability of the method for its intended use.

Sampling

The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.



1. Obscuration detector
2. Scattered beam
3. Direct beam
4. Fourier lens
5. Scattered light not collected by lens (4)
6. Particle ensemble
7. Light source laser
8. Beam processing unit
9. Working distance of lens (4)
10. Multi-element detector
11. Focal distance of lens (4)

Fig. 2.5.7-1: Example of a set-up of a laser light diffraction instrument

Evaluation of the Dispersion Procedure

The sample to be analyzed is inspected, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method, it is highly advisable to check that comminution of the particles does not occur and, conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change significantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g., crystallization, milling) of the material has changed, the applicability of the method must be verified (e.g., by microscopic comparison).

Sprays, aerosols, and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes, and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilizers) and/or mechanical forces (e.g., agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilization of the dispersion. For these liquid dispersions, a recirculating system consisting of an optical measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing is most commonly used. Nonrecirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers that apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol that is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free-flowing, coarser particles or granules, the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving, and the mass and percentage of removed material are reported. However, after presieving, note that the sample is no longer representative, unless proven otherwise.

Optimization of the Liquid Dispersion

Liquids, surfactants, and dispersing aids used to disperse powders must

- be transparent at the laser wavelength and practically free from air bubbles or particles;
- have a refractive index that differs from that of the test material;
- be a nonsolvent of the test material (pure liquid or prefiltered, saturated solution);
- not alter the size of the test materials (e.g., by solubility, solubility enhancement, or recrystallization effects);
- favor easy formation and stability of the dispersion;
- be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.); and
- possess a suitable viscosity to facilitate recirculation, stirring, and filtration.

Surfactants and/or dispersing aids are often used to wet the particles and to stabilize the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH, respectively, can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore, a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

Optimization of the Gas Dispersion

For sprays and dry powder dispersions, a compressed gas free from oil, water, and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

Determination of the Concentration Range

In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropriate concentration range for any typical sample of material.

NOTE—In different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g., obscuration, optical concentration, proportional number of total mass.

Determination of the Measuring Time

The time of measurement, the reading time of the detector, and the acquisition frequency is determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

Selection of an Appropriate Optical Model

Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, because small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about 0.01–0.1 i) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g., shape, surface roughness, and porosity) bear upon the final result.

Validation

Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision, and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate different components into a sample, nor is it possible to discriminate between agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly on the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled, robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as percentage RSD £ 10 per cent [n = 6] for any central value of the distribution (e.g., for x_{50}). Values at the sides of the distribution (e.g., x_{10} and x_{90}) are oriented towards less stringent acceptance criteria such as percentage RSD £ 15 per cent [n = 6]. Below 10 µm, these values must be doubled. Robustness may be tested during the selection and optimization of the dispersion media and forces. The change of the dispersing energy may be monitored by the change in the particle-size distribution.

Measurement

Precautions

The instructions given in the instrument manual are followed:

- never look into the direct path of the laser beam or its reflections;
- earth all instrument components to prevent ignition of solvents or dust explosions;
- check the instrument set-up (e.g., warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight); and
- in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent airflow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

Measurement of the Light Scattering of Dispersed Sample(s)

After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to subtract them later from the data obtained with the

sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation. The magnitude of the signal from each detector element depends upon the detection area, the light intensity, and the quantum efficiency. The coordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (the blank measurement) indicates the proportion of scattered light and hence the particle concentration.

Conversion of Scattering Pattern into Particle-Size Distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of deviations between measured and calculated scattering patterns (e.g., least squares), some constraints (e.g., non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

Replicates

The number of replicate measurements (with individual sample preparations) to be performed, depends on the required measurement precision. It is recommended to set this number in a substance-specific method.

Reporting of Results

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol x is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. Q3(x) denotes the volume fraction undersize at the particle size x. In a graphical representation, x is plotted on the abscissa and the dependent variable Q3 on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10 per cent, 50 per cent and 90 per cent (denoted as x_{10}, x_{50}, and x_{90}, respectively) are frequently used. x_{50} is also known as the median particle size. It is recognized that the symbol d is also widely used to designate the particle size, thus the symbol x may be replaced by d.

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. Because the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

Control of the Instrument Performance

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

Calibration

Laser diffraction systems, although assuming idealized properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument meets the requirements if the mean value of x_{50} from at least three independent measurements does not deviate by more than 3 per cent from the certified range of values of the certified reference material. The mean values for x_{10} and x_{90} must not deviate by more than 5 per cent from the certified range of values. Below 10 µm, these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, nonspherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analysis performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a

significant bias. The reason for this bias is that the different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same nonspherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

Qualification of the System

In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software, and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the x_{50} value does not deviate by more than 10 per cent from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g., x_{10} and x_{90}), then these values must not deviate by more than 15 per cent from the certified range of values. Below 10 µm, these values must be doubled.

NOTE—For calibration of the instrument, stricter requirements are laid down in the paragraph on Calibration.

2.5.8. Optical Microscopy: Particle Size by Microscopy

This method is suitable for counting and characterizing particles of 1 µm and greater. The lower limit is imposed by the resolving power of the microscope. With increased resolving power of microscope particles smaller than 1 µm can be detected and characterized. The upper limit is less definite and is determined by the increased difficulty associated with the characterization of larger particles. Various alternative techniques are available for particle characterization outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterizing particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

Apparatus. Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterization of the smallest particles to be classified in the sample under examination. The greatest numerical aperture of the objective should be sought for each magnification range. Polarizing filters in conjunction with suitable analyzers and retardation plates and colour filters of relatively narrow spectral transmission should be used with achromatic objectives in photomicrography. Condensers corrected for spherical aberration should be used in the microscope substage and with the lamp. The lamp should provide uniform and adjustable intensity of light over the entire field of view. The numerical aperture of the substage condenser should match that of the objective under the conditions of use and is affected by the actual aperture of the condenser diaphragm and by the presence of immersion oils.

Adjustment. The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements should be done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

Illumination. A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Kohler illumination is preferred. With coloured particles, choose the colour of the filters used so as to control the contrast and detail of the image.

Visual Characterization. The magnification and numerical aperture should be sufficiently high to allow adequate resolution of the images of the particles. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimized if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale should be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterize materials having a wide particle size distribution.

Photographic characterisation. If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing should be identical for

photographs of both the test sample and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

Preparation of the mount. The mounting medium will vary according to the physical properties of the test sample. Sufficient but not excessive contrast between the sample and the mounting medium is required to ensure adequate detail of the sample edge. The particles should rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care should be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

Crystallinity characterisation. The crystallinity of a material may be characterised to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the sample in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colours) and extinction positions when the microscope stage is revolved.

Limit test of particle size. Weigh a suitable quantity of the powder to be examined (for example, 10 to 100 mg), and suspend it in 10 ml of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

Particle size characterisation. The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterized must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined in Fig. 2.5.8-1.

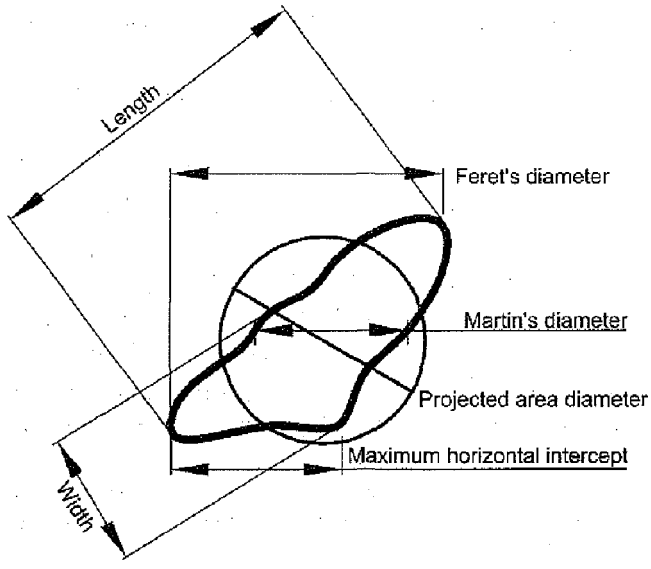


Fig. 2.5.8-1: Commonly used measurements of particle size

- *feret's diameter:* the distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale.
- *martin's diametre:* the diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas.
- *projected area diameter:* the diameter of a circle that has the same projected area as the particle.
- *length:* the longest dimension from edge to edge of a particle oriented parallel to the ocular scale.
- *width:* the longest dimension of the particle measured at right angles to the length.

Particle shape characterisation. For irregularly shaped particles, characterization of particle size must also include information on particle shape. The homogeneity of the powder should be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see Fig. 2.5.8-2).

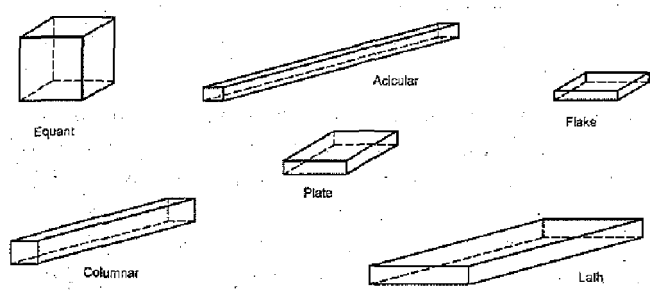


Fig. 2.5.8-2: Commonly used descriptions of particle shape

- *acicular:* slender, needle-like particle of similar width and thickness.
- *columnar:* long, thin particle with a width and thickness that are greater than those of an acicular particle.
- *flake:* thin, flat particle of similar length and width.
- *plate:* flat particles of similar length and width but with greater thickness than flakes.
- *lath:* long, thin, and blade-like particle.
- *equant:* particles of similar length, width, and thickness; both cubical and spherical particles are included.

General Observations. A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semisolid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

- *lamellar:* stacked plates.
- *aggregate:* mass of adhered particles.
- *agglomerate:* fused or cemented particles.
- *conglomerate:* mixture of two or more types of particles.
- *spherulite:* radial cluster.
- *drusy:* particle covered with tiny particles.

Particle condition may be described by the following terms:

- *edges:* angular, rounded, smooth, sharp, fractured.
- *optical:* colour (using proper colour-balancing filters), transparent, translucent, opaque
- *defects:* occlusions, inclusions.

Surface characteristics may be described as:

- *cracked:* partial split, break, or fissure.
- *smooth:* free of irregularities, roughness, or projections.
- *porous:* having openings or passageways.
- *rough:* bumpy, uneven, and not smooth.
- *pitted:* small indentations.

2.5.9. Particulate Contamination

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

Parenteral preparations including solutions constituted from sterile solids are expected to be free from particles of approximately 50 µm or more that can be observed by inspection with the unaided eye. However, parenteral preparations in containers that are labelled as containing 100 ml or more of a single-dose large volume injection intended for administration by intravenous infusion should comply with the limits of sub-visible particles prescribed in this test.

The test does not apply to multiple dose injections, to single dose small volume parenteral preparation and to parenteral solution constituted from sterile solids. For these preparations the following method of visual assessment is adequate. The test is intended to provide a simple procedure for the visual assessment of the quality of parenteral solutions as regards visible particles. Other validated methods may be used.

For visible particles

Apparatus. The apparatus consists of a viewing station comprising:

- a matt black panel of appropriate size held in a vertical position,
- a non-glare white panel of appropriate size held in a vertical position next to the black panel, and
- an adjustable lamp holder fitted with a suitable, shaded, white-light source and with a suitable light diffuser (a viewing illuminator containing two 13W fluorescent tubes, each 525 mm in length, is suitable). The intensity of illumination at the viewing point is maintained between 2000 lux to 3750 lux, for clear glass ampoules or higher for coloured glass and plastic containers.

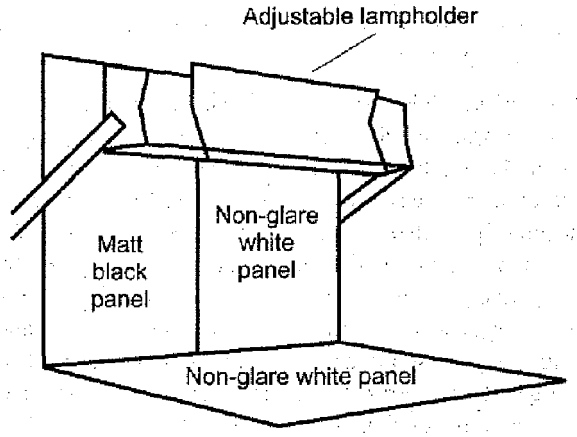


Fig. 2.5.9-1. Apparatus for visible particles

Method. Remove any labels on the container, wash and dry the outside. Gently swirl or invert the container ensuring that air bubbles are not introduced, and observe for about 5 seconds in front of the white panel. Repeat the procedure in front of the black panel. Record the presence of any particles Repeat the procedure for a further 19 containers.

Acceptance criteria

For liquid preparations. The preparation fails the test if one or more particles are found in more than one container.

For reconstituted solution of the lyophilized preparations. The preparation fails the test if particles are found in more than two containers.

For sub-visible particles

Two methods are specified, one involving the counting of particles viewed under a microscope and the other based on the count of particles causing light obscuration. Both methods are applied on small samples. It should be noted that the results obtained in examination of a single unit or group of units cannot be extrapolated with certainty to other units that have not been sampled or tested. However, it may be necessary to test some preparations by the light obscuration particle count test followed by the microscopic particle count test to reach a conclusion on conformance to the requirements. Therefore statistically sound sampling plans based upon a known set of given operational factors must be developed if valid inferences are to be drawn from the observed data to know the level of particulate contamination in a large group of units (such as a production batch).

Method 1. Microscopic particle count test

This method is suitable for revealing the presence of particles the longest axis or effective linear dimension of which is 10 µm or more.

Apparatus. A suitable binocular microscope, filter assembly and membrane filter for retention of particles.

The microscope is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage that can traverse the entire filtration area of the membrane filter, an internal illuminator for providing episcopic bright field illumination and an external focusable auxiliary illuminator for oblique illumination at an angle of 10° to 20°, and is adjusted to 100 ± 10 magnifications.

The eyepiece micrometer is a circular diameter graticule (Fig. 2.5.9-2) consisting of a large circle (field of view) divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10 µm increments. It should be calibrated using a standard stage micrometer grid. A relative error of the linear scale of the graticule within 2 per cent is acceptable. The large scale is designated the graticule field of view (GFOV).

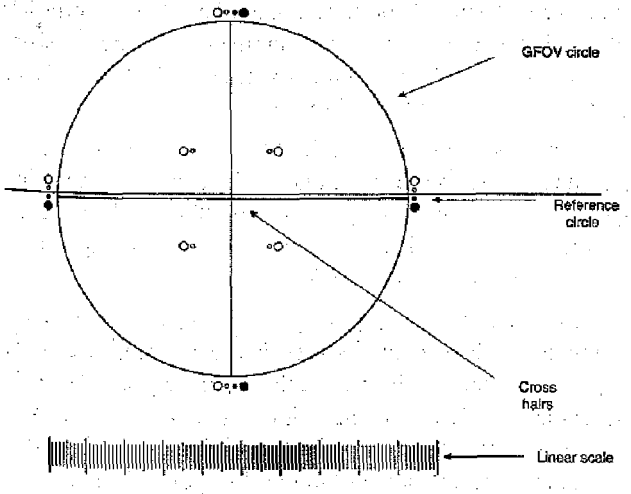


Fig. 2.5.9-2. Circular diameter graticule

The filter assembly consists of a filter holder of glass or metal and is connected to a vacuum source and a suitable membrane filter. The filter is black or dark grey in colour, is gridded or non-gridded and has a pore size of 1.0 µm or less.

Precautions during testing. It is important that the test is carried out under conditions that prevent the introduction of extraneous particulate contamination. The procedures should be done in a laminar air-flow cabinet or hood (of the horizontal type), equipped with HEPA (high efficiency particulate air) filters, preferably in a controlled-air environment.

Prior to carrying out the test the cabinet should be cleaned with an appropriate solvent. The glassware and filter assembly used, except for the membrane filter, should be washed carefully with a warm detergent solution and then rinsed with plenty of water to remove all traces of detergent. Immediately before use, both sides of the membrane filter and the equipment should be rinsed with particle-free water that is filtered through membrane with the pore- size of 0.22 µm. Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out. The efficacy of these measures and the suitability of the environment should be checked by carrying out the following test.

Determine the particulate contamination of a 50-ml volume of *particle-free water* as described below. If more than 20 particles 10 µm or larger in size or if more than 5 particles 25 µm or larger in size are present within the filtration area the preparatory steps taken must be repeated until the right conditions for testing are achieved.

Method. Invert the container of the preparation under examination 20 times successively in order to mix the contents, If necessary, cautiously removing the sealing closure. Wash the outer surface of the container with a jet of particle-free water and remove the closure carefully, avoiding contamination of the contents.

For large-volume parenterals, single units should be tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units should be combined in a clean container. Where the volume of liquid in a container is very small, the test solution may be prepared by mixing the contents of a suitable number of containers and diluting to 25 ml with *particle-free water* or with an appropriate solvent without contamination of particles when particle free water is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually.

Powders for parenteral use should be constituted with *particle-free* water or with an appropriate solvent without contamination of particles when particle free water is not suitable.

In general, the number of test samples must be adequate to provide a statistically sound assessment of the particulate contamination. For large-volume parenterals or for small-volume parenterals having a volume of 25 ml or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Using flat-ended forceps, carefully remove a colour contrast grid membrane filter from its container. Wash both sides of the membrane with a stream of particle-free water, starting at the top of the non-gridded side (unprinted), sweeping the stream back and forth across the surface and working slowly from top to bottom; repeat the process on the gridded side. Place the membrane with the grid side up on the filter base and install the filtering funnel on the base without sliding the funnel over the membrane filter. Invert the assembled unit and wash the inside of the funnel for about 10 seconds with a jet of *particle-free water*. Allow the water to drain and place the unit on the filter flask.

Transfer to the filtration funnel the total volume of the pooled solution or of a single unit, allow to stand for a minute, apply the vacuum and filter. If necessary, the transfer may be done in portions until the entire volume is filtered. After the last addition of solution, start rinsing the inner walls of the filter holder with a jet of *particle-free water*. Direct the jet of water in such a manner as to wash the walls of the funnel free from any particles that may have become lodged on the walls but avoid directing the stream onto the filter surface. Maintain the vacuum until the surface of the membrane filter is free from liquid. Remove the membrane filter with flat-ended forceps, place it with the gridded surface up in a Petri dish or slide and allow it to dry in air with the cover slightly a jar. After the filter has been dried, place the dish on the stage of the microscope; scan the entire membrane filter under reflected light. Count the number of particles that are equal to or greater than 10 µm, the number of particles equal to or greater than 25 µm and the particles equal to or greater than 50 µm. Alternatively, partial filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined

Sizing of the particles using the circular diameter graticule may be done by transforming mentally the image of each particle into a circle and then comparing it to the 10 µm and 25 µm graticule reference circles Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles. Dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

NOTE — Do not count or size amorphous, semi-liquid, or otherwise morphologically indistinct materials that have the appearance of a stain or show little or no surface relief and present a gelatinous or film-like appearance. In such cases the interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count test.

Limits. Table 1

Sample	Particle size in µm (equal to or greater than)	Maximum number of particles
Preparations in containers with nominal content of more than 100 ml	10	average in the units tested 12 per ml
	25	2 per ml
Preparations in containers with nominal content of 100 ml	10	3000 per container
	25	300 per container
Less than 100 ml	10	3000 per container
	25	300 per container

The preparation meets the requirements of the test if it contains particles within the maximum limits shown in Table 1.

Method 2. Light obscuration particle count test

This method is not suitable for preparations with reduced clarity or increased viscosity such as emulsions, colloids, liposomal preparations and products that produce air or gas bubbles when drawn into the sensor.

Precautions during testing. The same general precautions as for Method 1 apply.

Apparatus. A suitable particle counter based on the principle of light blockage and capable of automatic counting and sizing of particles. The manufacturer's instructions for installation and operation should be followed.

The instrument should be calibrated with suitable certified reference materials consisting of dispersions or spherical particles of size in the range 10 µm to 25 µm. The materials should be dispersed in particle-free water, taking care to prevent aggregation of particles.

Method. Invert the container of the preparation under examination 20 times successively in order to mix the contents If necessary, cautiously remove the sealing closure. Wash the outer surface of the container with a jet of *particle-free water* and remove the closure carefully, avoiding contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating

For large-volume parenterals, single units should be tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units should be combined in a clean container to obtain a volume of not less than 25 ml. Where the volume of liquid in a container is very small, the test solution may be prepared by mixing the contents of a suitable number of containers and diluting to 25 ml with *particle-free water* or with an appropriate solvent without contamination of particles when particle free water is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually.

Powders for parenteral use should be constituted with *particle-free water*, or with an appropriate solvent without contamination of particles when particle free water is not suitable. The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25ml or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Remove 4 portions, each of not less than 5 ml, and count the number of particles equal to or greater than 10 µm and 25 µm. Ignore the result obtained for the first portion, and calculate the average number of particles in the preparation under examination.

Limits.

Table 2

Sample	Particle size in µm (equal to or greater than)	Maximum number of particles
Preparations in containers with nominal content of more than 100 ml	10	average in the units tested
	25	25 per ml
		3 per ml
Preparations in containers with nominal content of 100 ml	10	6000 per container
	25	600 per container
Less than 100 ml	10	6000 per container
	25	600 per container

The preparation meets the requirements of the test if it contains particles within the maximum limits shown in Table 2. If the average number of particles exceeds these limits, test the preparation by Method 1.

2.5.10. Validation of Analytical Procedures

Validation of an analytical procedure is performed in order to demonstrate that the procedure is suitable for its intended use. Validation is performed in order to show that the result(s) generated by a particular analytical procedure are reliable and accurate.

Types of procedures to be validated

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

Type of analytical procedure	Identification	Testing of Impurities		Assay - dissolution (measurement only) - content/potency
characteristics		quantitat.	limit	
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm. Precision	-	+(1)	-	+(1)
Specificity (2)	+	+	+	+
Detection Limit	-	-(3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated.

+ signifies that this characteristic is normally evaluated.

(1) in cases where reproducibility has been performed, intermediate precision is not needed.

(2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(3) may be needed in some cases.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Specificity may often be expressed as the degree of bias of test results obtained by analysis of samples containing added impurities, degradation products, related chemical compounds, or placebo ingredients when compared to test results without added substances.

Specificity is usually demonstrated by measuring the response of the sample matrix and any expected or known species (for example excipients, impurities or degradation products). It would normally be expected that no significant response would be obtained that interferes with the measurement of the analyte(s). However it is not always possible that an analytical procedure is specific for a particular analyte. In this instance a combination of two or more analytical procedures may be necessary to achieve the required discrimination.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Linearity is usually demonstrated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be calculated. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

A minimum of five concentrations is recommended. Other approaches should be justified.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure.

Accuracy is usually demonstrated by adding known amounts of analyte(s) to the sample matrix and determining the measured result using the analytical procedure. The recovery of measured against actual amounts is then calculated. Usually a minimum of three determinations at each of three concentrations across the intended range is recommended.

Accuracy may also be demonstrated by the method of standard additions, or by cross-correlation of results with a second, independent, procedure. Accuracy may be inferred once precision, linearity and specificity have been established.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability (Intra-Assay Precision)

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision.

Repeatability is usually demonstrated by repeated measurements of a single sample (e.g. use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment). A minimum of three determinations at each of three concentrations across the intended range, or a minimum of six determinations at the test concentration is recommended.

Intermediate Precision

Intermediate precision expresses within-laboratory variations: different days, different analysts or equipment, etc.

Intermediate precision is usually demonstrated by repeated measurements of the sample used in the repeatability experiment within the same laboratory. Usually the repeatability experiment is repeated on the same sample by a different analyst, on a different day, using different equipment if possible.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology).

Reproducibility is usually demonstrated by means of an inter-laboratory trial.

Detection Limit

The detection limit of an analytical procedure is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated as an exact value.

The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

The detection limit is usually demonstrated by measuring low concentrations of the analyte and showing that a response is obtained.

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

It is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

The quantitation limit is usually demonstrated by measuring low concentrations of the analyte and showing that a repeatable response is obtained.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Robustness is usually demonstrated by making small deliberate changes to one of the operating parameters of the method, analysing samples and comparing the results to those obtained using the prescribed method.

Range

The range of an analytical method is the interval between the upper and lower concentration (amounts) of analyte (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

For assays the range is usually not less than 80 to 120 per cent of the test concentration.

For determination of content uniformity the range is usually not less than 70 to 130 per cent of the test concentration.

For determination of impurities the range is usually not less than the reporting limit of the impurity to 120 per cent of the specification.

For dissolution testing the range is usually ± 20 per cent over the expected concentrations.

Range is usually demonstrated by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range.

2.5.11. Polymorphism

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state. It is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulphur), the term allotropy is used instead of polymorphism.

The term pseudopolymorphism is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include compounds where the solvent is trapped in the matrix in variable proportions. However the term pseudopolymorphism is ambiguous because of its use in different circumstances. It is therefore preferable to use only the terms "solvates" and "hydrates".

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, occurence of solvates, allotropy or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At normal temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. The change from one phase to another is a univariate equilibrium, so that at a given pressure this state is characterised by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

Different crystalline forms or solvates may be produced by varying the crystallisation conditions (temperature, pressure, solvent, concentration, rate of crystallisation, seeding of the crystallisation medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism:

- X-ray diffraction of powders,

- X-ray diffraction of single crystals,
- Thermal analysis (differential scanning calorimetry, thermogravimetry, thermomicroscopy),
- Microcalorimetry,
- Moisture absorption analysis,
- Optical and electronic microscopy,
- Solid-state nuclear magnetic resonance,
- Infrared absorption spectrophotometry,
- Raman spectrometry,
- Measurement of solubility and intrinsic dissolution rate,
- Density measurement.

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise solvates are less soluble in their solvent than unsolvated forms.

2.6. TESTS ON HERBAL PRODUCTS

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2.6.1. Foreign Organic Matter

Foreign organic matter is the material consisting of any or all of the following.

(1) Parts of the organ or organs from which the drug is derived other than the parts named in the definition and description or for which the limit is prescribed in the individual monograph.

(2) Any organs other than those named in the definition and description.

(3) Matter not coming from the source plant.

(4) Moulds, insects or other animal contamination.

Method

Weigh 100 to 500 g, or the quantity specified in the individual monograph, of the *original sample* and spread it out in a thin layer. Inspect the sample with the unaided eye or with the use of a 6x lens and separate the foreign organic matter manually as completely as possible. Weigh and determine the percentage of foreign organic matter from the weight of the drug taken. Use the maximum quantity of sample for coarse or bulky drugs.

2.6.2. Ethanol-Soluble Extractive

Macerate 5 g of the air-dried drug, coarsely powdered, with 100 ml of *ethanol* of the specified strength in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of ethanol, evaporate 25 ml of the filtrate to dryness in a tared flat-bottomed shallow dish, dry at 105° and weigh. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

2.6.3. Water-Soluble Extractive

Method I

Proceed as directed for the determination of Ethanol-soluble Extractive, using *chloroform water* instead of *ethanol*.

Method II

Add 5 g to 50 ml of *water* at 80° in a stoppered flask. Shake well and allow to stand for 10 minutes, cool, add 2 g of *kieselguhr* and filter. Transfer 5 ml of the filtrate to a tared evaporating dish, 7.5 cm in diameter, evaporate the solvent on a water-bath, continue drying for 30 minutes, finally dry in a steam oven for 2 hours and weigh the residue. Calculate the percentage of water-soluble extractive with reference to the air-dried drug.

2.6.4. Complete Extraction of Alkaloids

Complete extraction is indicated by the following tests.

When extracting with an aqueous or alcoholic liquid — After extracting at least 3 times with the liquid, add to a few drops of the next portion, after acidifying with *2 M hydrochloric acid* if necessary, 0.05 ml of *potassium mercuri-iodide solution* or, for solanaceous alkaloids, 0.05 ml of *potassium iodobismuthate solution*; no precipitate or turbidity is produced.

When extracting with an immiscible solvent — After extracting at least 3 times with the solvent, add to a few drops of the next portion 1 to 2 ml of *0.1 M hydrochloric acid*, remove the organic solvent by evaporation, transfer the aqueous residue to a test-tube, and add 0.05 ml of *potassium mercuri-iodide solution* or, for solanaceous alkaloids, 0.05 ml of *potassium iodobismuthate solution* or, for emetine, 0.05 ml of *iodine solution*; not more than a very faint opalescence is produced.

Continuous extraction —After percolating for at least 2 hours, collect 1 to 2 ml of the effluent and carry out the procedure described under '***When extracting with an aqueous or alcoholic liquid***' or '***When extracting with an immiscible solvent***' as appropriate.

2.6.5. Total Solids

The term 'total solids' is applied to the residue obtained when the prescribed amount of the preparation is dried to constant weight under the conditions specified below.

Apparatus

Shallow, flat-bottomed, flanged dishes, about 75 mm in diameter and about 25 mm deep, made of nickel or other suitable metal of high heat conductivity and which is not affected by boiling *water*.

Method

Weigh accurately or measure an accurate quantity of the substance under examination stated in the individual monograph, place in a tared dish, evaporate at as low a temperature as possible until the solvent is removed and heat on a water-bath until the residue is apparently dry. Transfer to an oven and dry to constant weight at 105°, unless otherwise stated in the monograph. Owing to the hygroscopic nature of certain residues, it may be necessary to use dishes provided with well-fitting covers and to cool in a desiccator.

2.6.6. Evaluation of Herbs and Processed Herbs

India Pharmacopoeia (IP) specifies and describes in individual monographs tests for identification of herbs and processed

herbs. These tests involve morphological, microscopic, organoleptic and chromatography profile as applicable to individual monograph entity. These tests primarily are based on pharmacognostic characterization and are aimed to assist in botanical identification of the herb. Wherever possible the monograph also provides tests to differentiate from potential adulterants or substitutes or closely related herbs. IP has also provided testing for DNA barcode as a confirmatory test of identity in case of difficulty or dispute for correct identity basis tests mentioned above. Currently DNA barcode test is not mandatory if identity is possible on the basis of data of the other tests mentioned above. Monographs on herbs cover different parts of the plants namely bark, root, flower and their parts, whole plant, fruits and their parts, underground stems, leaves, woods, seeds and unorganized herbs and any other specific part of the plant which becomes the ingredient for use in different formats for external or internal consumption for animals and humans. These may also be the starting material for obtaining processed herbs, phytopharmaceuticals or purified chemical compounds. Botanical identity confirmation of such part or plants forms a mandatory requirement in the monograph and is a requirement for quality of products made from them. Monographs in IP cover either organized herb or unorganized herbs. Organized herbs contain tissues of the plant or part of the plant and powdered herbs. Unorganized herbs are produce of plants such as gums, mucilage, resins, juices and latex. Concentrated extracts, fractionated extracts and distilled or expressed oils are also unorganized herbs. Such unorganized herbs have a fairly uniform composition and are devoid of any cellular structure.

IP suggests comparison of the test substance with samples of herbs whose authenticity has been confirmed kept as reference material or to a herbarium wherever possible. IP also provides for use of Pharmacopoeial Botanical Reference Substance (BRS) as part of monograph for comparison while undertaking chromatographic profile test.

Tests for identity by macroscopy is conducted through tests for colour, odour, taste, size, form, fracture, marking and other pharmacognostic features of the underground material received and collected as a sample for evaluation. Except for ready ground material herbs are received in their whole, broken to small sizes or in crumpled form for further use. Where prescribed tests are to be conducted as described below which provide broad methods.

Organoleptic Evaluation

*Colour**. Examine the color of the sample in diffused day light or artificial light, similar to day-light. For herbs like barks and leaves, colors on the outer and inner side should be noted separately. The colors are expressed as primary or secondary colors. In case of mixed colors i.e. yellow and brown, if brown is predominant the color is expressed as 'yellowish brown' and if yellow is predominant express the color as 'brownish yellow'.

*Odour**. If a volatile component is present in the herb, it gives an odour otherwise the herb is odourless. If the material is expected to be innocuous, place a small portion of the sample in the palm of the hand or in a beaker of suitable size and slowly and repeatedly inhale the air over the material. If no distinct odour is perceptible, crush the sample between the thumb and index finger or between the palms of the hands using gentle pressure. If the material is known to be dangerous, crush by mechanical means and then pour a small quantity of boiling water onto the crushed sample in a beaker. First, determine the strength of the odour (none, weak, distinct, strong) and then the odour sensation (aromatic, fruity, musty, mouldy, rancid, etc.). A direct comparison of the odour with a defined substance is advisable (e.g. peppermint should have an odour similar to menthol, cloves should have an odour similar to eugenol).

*Taste**. It is known that the odour also contributes to the taste. 'Taste' is used as criteria for evaluation of some herbs containing bitter or pungent compounds, to determine the total bitterness as 'bitterness value'. The threshold of bitter concentration of the extract of the herb is compared with a dilute solution of *quinine hydrochloride*. The bitterness value is expressed in unit's equivalent to the bitterness of solution containing about 1 g of *quinine hydrochloride* in 2000 ml. Similarly for herbs with pungent taste it is possible to grade them based on heat value which is compared to the pungent and hot effect given by a standard solution of capsaicin. Indian Standard (IS) Specification provides details of heat value determination to not only qualitatively determine the taste but also quantitatively estimate the same. Taste is specified by using terms such as sweet, salty, sour, pungent, slimy, bitter, acrid, warm and astringent.

Size. Measure the length, width and thickness of crude herbal material and report values in millimeter or centimeter.

a. *For large samples*. Take measurements directly. Place the sample on the scale measure the length, width and thickness.

b. *For very small samples*. Like small seeds and fruits, arrange about 10 samples in a straight-line lengthwise measure and take the average as length. For width, arrange 10 samples width wise in a straight-line measure and take the average.

c. *Herbs inshriveled or matted condition*. These herbs need re-hydration eg: thin leaves (*Digitalis purpurea*),

**Keeping in mind safety and potential risk tests for odour and taste are being mentioned in monographs only for information purpose and not as tests for quality as part of monograph. These tests to be undertaken by trained personnel with adequate safety precautions when prescribed as a part of the monograph.*

flowering tops (*Datura metel*), flowers (*pyrethrum*) etc., in this condition the size and shape cannot be studied. Care should be taken not to break the mass while rehydrating the dried samples for evaluation

Take the sample of drug that is to be re-hydrated. Soak the herb mass in *water* for a few minutes. Remove the excess *water* using blotting paper. Expand the leaves with the fingers on a blotting paper and carefully press and remove the excess *water*.

OR

Pour some *water* at the bottom of a dessicator, keep the herb on a blotting paper in the middle. Close the desiccator and leave it over night. Next day take the herb; spread them on a blotting paper. Press and preserve it for morphological and microscopical studies.

OR

Keep the dry herb between wet blotting papers for few hours and carefully spread them out when they are soft and study.

*Form**. Depending on the different morphological group, the form may be whole, separated, broken, cut, peeled and unpeeled.

Surface characteristics (markings), texture and fracture characteristics: Examine the untreated sample. A magnifying lens (6x to 10x) may be used. Wetting with *water*/reagents as required, may be necessary to observe the characteristics of a wet surface. Touch the material to determine if it is soft with hand; bend and rupture it to obtain information on brittleness and appearance of the fracture- fibrous, smooth and granular.

Document the overall form of the sample under examination through photography.

The following section provides broad information on pharmacognostic description to aid in analysis of samples.

Woods

Wood normally is the central portion of the stem or the root of the perennial trees. Commercial supplies of wood may be in large and dense (Sandal wood) pieces either horizontally or transversely cut pieces or as shavings, raspings and chips (Quassia wood) to aid drying.

Wood is differentiated as heartwood or duramen and the sapwood or alburnum consisting of the conducting tissues. Heartwood is the dead tissue darker in colour and adds only to the mechanical strength. Tannins and resins are deposited in this tissue. Sapwood forms the drug.Terms that describe different characteristics of fracture are ring, porous and diffused. Markings are described with terms like annual rings, straight, grained or interlocked grains. Woods may be either heavy or light in nature.

Roots and under-ground modifications of stem

Herbs both roots and under-ground modifications of stem include rhizomes, corm, stolon, tubers and annular roots.

Broad guidance is provided related to roots or modified underground stems.

Table 1– Herbs from roots, stems and their modifications

Part	Examples
Roots only	*Aconitum napellus* (tuberous), *Atropa bella-donna*, *Rauvolfia serpentina*, *Carapichea ipecacuanha* (annular roots)
Stem modifications	*Colchicum autumnale* (corm), *Dioscorea alata* (tuber), *Zingiber officinale* (rhizome), *Curcuma longa* (rhizome), *Rheum* (rhizome)
Herbs containing root and rhizome	Gentian, *Picrorhiza kurroa*, *Podophyllum peltatum*, *Valeriana officinalis*
Herbs containing root and stolon	*Glycyrrhiza glabra*

Table 2 – Morphological difference between stem and root

Character	Stem & Underground stem modifications	Root
Position	Aerial	Underground
Growth	Vertical, Horizontal, or Oblique	Vertical
Form	Straight	Tortuous
Leaves, scale leaves or scars	Present	Absent
Node and inter nodes	Present	Absent
Terminal or axillary buds	Present	Absent
Centre (dicotyledons)	Soft (pith)	Hard

Seeds

The Seed is represented by embryo (plumule, radical and one or two cotyledons), endosperm (storage tissue containing protein and fat), nucellus (perisperm containing starch), seed coat, coverings of the ovule (testa and tegmen), pericarp (separate or fuse with the seed coat).There are two types of seeds: Endospermous or Albuminous and Non- endospermous or Exalbuminous.

Table 3 – Types of seeds

Endospermous or Albuminous	Non-endospermous or Exalbuminous
Food storage outside embryo	Food storage in the embryo
The embryo is small and endosperm remains as a permanent food storage tissue of the seed	The growing embryo completely uses up the endosperm resulting in the embryo occupying the complete seed cavity. A thin layer may be present some times as in *Prunus amygdalus*(almond) seeds
i. Herbs as only endosperm example *Strychnos nux-vomica* and *Plantago* (isphagula)	Herbs as oily cotyledons: example *Amygdalus communis* (bitter almond), *Arachis hypogaea* (groundnut) and *Brassica nigra* (mustard) seed
ii. Herbs as endosperm and perisperm example *Myristica fragrans* (nutmeg), *Elettaria cardamomum* (cardamom) and *Areca catechu*	

Kernel forms the herb in many seeds. It is embryo with cotyledons covered with a thin testa. The markings of the seeds can be seen on the kernel as depressions or grooves indicating the positions on the seed. Different parts of ovule are seen on the seed: seed coat, the outer and inner integuments of the ovule form the testa and tegmen of the seed. The characteristics of the seed coat help in the identification of the seed at species, genus or family level.

Hilum, the point of attachment of the stalk or funicle; micropyle, the small opening at the apex of the integuments; chalaza, the point not visible but can be seen as a depression in some seeds; Raphe, the extension of funicle growing along side of the ovule,in some seeds there are outgrowths of tissues from different parts; Aril, covering arising from the funicle end, example mace in *Myristica fragrans*; Arillode, covering arising from the micropyle end as in *Elettaria cardamomum*; Caruncle, fleshy growth from the micropyle end as in *Ricinus communis* (Castor) seed; Strophiole, along the line of raphe in *Colchicum autumnale* seed; Wing, the extension of testa as a fold in *Strophanthus kombe*; Awn, elongated feathery extension as in *Strophanthus kombe*.

Barks

Barks are the outermost covering of stems and roots in the dicot plants. 'Botanical bark' consists of the cork and cork cambium only. The 'Pharmacognostical' bark consists of all the tissues present after the secondary growth, starting from the cork outside to the cambium inside.

If all the tissues are present, bark is known as the 'whole bark'. The cork and the cork cambium forms 'outer bark'. The secondary cortex and the primary cortex and endodermis together form the 'middle bark.' The pericycle, primary and secondary phloem forms the inner bark along with the medullary rays. In some barks, the outer and the inner barks are scrapped off as these tissues do not contain any therapeutically active constituent's example *Cinnamomum zeylanicum* and *Quillaja saponaria*.

I. **Shape.** The shape of the pieces depend upon the type of incision made at the time of its removal from the tree trunk and also upon the nature of shrinkage during drying.

i. *Flat.* Large pieces of bark often dried under pressure becomes flat in appearance example *Tecomella undulata* and *Boswellia serrata*.

ii. *Curved.* The bark is slightly concave towards the inside example *Stereospermum suaveolens*.

iii. *Re-curved.* The concavity is on the outside example *Holarrhenaanti dysenterica* and *Wrightia tinctoria*.

Table 4 – Type of ovule and presence/absence of Raphe

Type of ovule	Raphe	Example
Orthotropous ovule	Raphe is absent	*Strychnos nux-vomica* and *Plantago*
Amphitropous ovule	Raphe is short; half the length of seed	*Strophanthus kombe* and *Cascabela thevetia*
Anatropous ovule	Raphe is as long as seed	*Prunus amygdalus*, *Linum usitatissimum*, *Myristica fragrans* and *Elettaria cardamomum*
Campylotropous ovule	Raphe is absent	Solanaceous seeds

iv. *Channeled*. The curvature on the inside is deep enough to form a deep trough or channel example *Myrica esculenta*.

v. *Quill*. The curvature is present and one edge overlaps the other example *Spondia spinnata*.

vi. *Double quill*. Each edge is rolled independently into a quill example *Cinnamomum zeylanicum*.

vii. *Compound Quill*. When quills are packed one inside the other.

2. **Outer Surface**. The colour and condition of the outer surface gives a characteristic identification to the bark.

i. *Scaly*. Layers of dead phloem alternating with bands of cork frequently form a massive external covering of the bark (rhytidoma) and gives a scaly appearance.

ii. *Smooth*. When the cork is evenly developed.

iii. *Lenticels*. The smooth surface of bark is frequently marked with lenticels, which are commonly elongated and generally placed transversely to the long axis of the bark.

iv. *Cracks and Fissures*. Due to the continuous increase in girth of tree and lack of elasticity of dead tissues, cracks and fissures are formed and are often characteristic e.g. with clean cut edges as in *Cinchona succirubra* but thickened, uncurved edges as in *Cinchona officinalis*.

v. *Warts*. On older barks small circular dusty patches frequently develop in the cork.

vi. *Wrinkle*. The shrinkage of barks during drying occurs generally transversely because the fibres prevent the shrinkage in longitudinal direction and the greater shrinkage of the softer tissues results in the formation of wrinkles.

vii. *Furrows*. If troughs between the wrinkles are very wide.

viii. *Presence of Epiphytes*. The additional character is provided by the presence oflichens or bryophytes, including both liverworts and mosses.

3. **Inner Surface**. The color and condition of the inner surface of the bark also has diagnostic value.

i. *Striations*. The shrinkage results in the formation of parallel longitudinal ridges which are sometimes very fine or quite coarse.

ii. *Corrugations*. The longitudinal shrinkage produces parallel transverse wrinkles and is termed as corrugations.

iii. *Network of raised lines*. Very unequal shrinkage of portions of differing hardness associated with an anastomosis of the fibre strands example *Gmelina arborea*.

iv. *Smooth*. When the innermost tissues are uniformly soft, on drying, they form a firm dense layer giving a very hard smooth inner surface, example *Myrica nagi*.

4. **Fracture**. The appearance and behavior of the bark when broken across transversely, subjected to sufficient pressure is known as fracture.

i. *Complete*. breaking clean across.

ii. *Incomplete*. breaking only part of the way across.

iii. *Fibrous*. breaking that the broken ends have a jagged appearance from projecting fibres or fibro-vascular bundles.

iv. *Flexible*. breaking only by tearing or twisting.

v. *Brittle*. crumbling readily into fragments.

vi. *Granular*. peculiar, grain-like appearance of the broken surface.

vii. *Hard*. exhibiting a compact surface when broken.

viii. *Horny*. exhibiting a hard, horn-like surface.

ix. *Mealy*. breaking readily across the drug and exhibiting a starchy, broken surface.

x. *Resinous*. exhibiting a smooth, glossy, broken surface.

xi. *Conchoidal*. resembling the resinous fracture, but the surface is characteristically curved in convex and concave fashion.

xii. *Sharp*. breaking straight across.

xiii. *Short*. breaking quickly.

xiv. *Smooth*. breaking with a smooth surface.

xv. *Splintery*. breaking irregularly across into pieces with larger and smaller projecting edges and splinters.

xvi. *Tough*. breaking only after twisting or tearing.

xvii. *Uneven*. breaking in such a way as to exhibit irregularly broken surfaces.

xviii. *Waxy*. exhibiting peculiar, dull, granular, broken surfaces.

xix. *Weak*. breaking quickly with little effort.

xx. *Laminated*. the fractured region breaks into stratified layers.

Fruits

Fruits develop from the fertilized ovary. The ovary wall develops as pericarp of the fruit and the ovules as seeds. The crude drugs may be derived from the whole fruit including the seeds, like umbelliferous fruits, *Piper nigrum*, *Cassia angustifolia* (senna) fruits, or the outer part of the fruit 'pericarp', example *Citrus limon* (lemon peel).

The pericarp is divided into outer epicarp, the middle mesocarp and the inner endocarp. The mesocarp contains the vascular tissue. The endocarp may fuse with the seed coat as in *Foeniculum vulgare* and *Piper nigrum*, or form a hard cover for the seed as in *Prunus amygdalus* and *Myristica fragrans*.

Table 5 – Classification of fruits

Drug	Part	Type of fruit	Characteristics
Black pepper	Mature unripe fruit	Sorosis	From catkin
Capsicum	Ripe fruit	Berry	Persistent calyx
Lemon peel	Epicarp and mespcarp	Outer part of hesperidium	Oil glands
Myrobalan	Epicarp and mespcarp	Drupe	Hard endocarp
Star anise	Follicles	Composite fruit (etaerio of follicles)	Apocarpous ovary
Umbelliferous fruits	Mature fruit	Cremocarps	Sepals, stylopod

The fruit also shows the number of locules from which it develops. The fruit developing from epigynous (superior) ovary may have some floral parts attached to it, persistent calyx at the base of the fruit, e.g. *Capsicum annuum*, *Datura metel*. In hypogynous (inferior) ovary; calyx and stylopod are seen on top of the fruit as in umbelliferous fruits.

The arrangement of ovules/seeds on the placenta is known as 'placentation'. Dehiscence of the fruit and its carpels are some of the points to be noted in the identification of fruits.

Leaves

Leaves are the appendages of the stem. They are green in colour due to chlorophyll and traversed by supporting or conducting tissue, the veins. 'Lamina' or 'blade' is the expanded portion attached to the stem by a stalk or petiole. It has two surfaces: the one closer to the internode or faces upwards is adaxial surface and the one lower is abaxial. The leaf with petiole is petiolateand without, it is sessile. In some leaves, stipules are present at the base of the leaf called as stipulate, if the stipules are absent, called as exstipulate.

Leaves may be simple or compound. The leaf makes an angle with the stem, 'axil'. Usually an axillary bud or a branch can be seen in this axil of the leaf.

Compound leaf. The lamina is divided into smaller units 'leaflets', all lying in the same plane. A leaf with even pairs of leaflets is a paripinnate leaf and a leaf with even pairs of leaflets but with one odd leaflet at the tip is an imparipinnate leaf. The leaflets usually have asymmetric bases.

Venation. The conducting tissue from the stem, through the petiole, extends as a midrib (1°) into the lamina and gives rise to secondary (2°), tertiary (3°) and smaller veins going up to (7°). Dicotyledons show network of veins, the reticulate venation and Monocotyledons show parallel venation in which all veins are same and run parallel to each other.

In some leaves with reticulate venation, the secondary veins join the upper veins near the margin forming a continuous line of vein at the margin, known as anastomisation

Important identification features in the study of leaves are the shape, apex, margin, base, texture, surface and the projection of the midrib towards upper or lower surfaces, the angles at which the secondary veins leaves the midrib and anastomisation.

The terms describing the shape, apex, margin, base, etc., are the same for leaflets also. Texture is described by the terms membraneous, chartaceous, thick, stiff, coriaceous, leathery and tough. Surface by terms glabrous, pubescent and hirsute.

Flowers

Few flowers or their parts are used as herbs. Calyx, corolla androecium and gynoecium are the four main parts in a flower. These are arranged on top of the vegetative axis, the thalamus or torus in a definite pattern. Pedicel or stalk, bract and bractiole are the other minor parts.

Study the flower as it is if 'firm'. Rehdydrate the flower if in crumpled form. Look for other parts associated with the flower like pedicels, peduncle, etc. Note the colour, odour, taste and size. Study the types of inflorescence.

Calyx. Sepals: Note the number, colour, shape and aestivation.

Corolla. Petals: Note the number, colour, shape and aestivation. Study the aestivation either in the flower or in the bud.

Androecium. Stamen: Note the number, size and colour and their arrangement with respect to the petals, their grouping and attachments. Study the filament, anther lobes, their dehiscence and pollen grains.

Gynoecium: Study the ovary, style and stigma. Note any nectar glands at the base of the ovary. Study the position of ovary whether inferior, superior or half superior and half inferior.

Entire Organisms

These herbs contain the whole aerial parts including stems of particular diameter, leaves, buds, flowers, fruits and roots.

The respective morphological groups may be referred to identify the specified parts of the herb. Few examples of entire

organisms are as follows. Cannabis (*Cannabis sativa*), Ergot (*Claviceps purpurea*), Ephedra (*Ephedra gerardiana*), Lobelia (*Lobelia nicotiana*), Belladonna (*Atropa belladonna*), Hyoscyamus (*Hyoscyamus niger*), Stramonium (*Datura stramonium*), Taxus (*Taxus brevifolia*).

2.6.7. Microscopic Evaluation of Herbs and Processed Herbs

Microscopic evaluation is a definite method which helps in the identification of crude herbs/ substitutes/ adulterants in addition to macroscopic evaluation. In a group of herbs containing the same chemical compound like *hyoscyamine* in *Belladonna*, *Hyoscyamus* and *Stramonium*, microscopic character like type of crystals gives the correct identity of the herb. The microscopic features of *Senna* and *Digitalis* as leaves are the same. But the isobilateral leaf structure, unicellular, warty trichomes, presence of crystals differentiates *Senna* from *Digitalis* which has dorsiventral leaf structure, multicellular collapsed trichomes and absence of crystals.

The evaluation includes: Microscopic sections of the herb, Powder study of processed herbs, Quantitative microscopy of herbs, Quantitative microanalysis and Microchemistry of herbs and processed herbs. Any additional useful information for preparation or analysis should be included in the text procedure for individual plant materials for example leaf constants: stomatal number and stomatal index, vein-islet and veinlet termination number and palisade ratio.

Broad guidance

a) Microscopic sections of the herb

In a particular morphological group of herbs, the microscopic/ histological characters are mostly similar with a few specific differences. These specific differences form the basis for identification of the crude herb. Sections of the herbs are studied for the arrangement of general tissues and specialized tissues which are specific for the particular herb and are compared with standard literature to identify the herb. Any tissue other than the tissues of the authentic herb indicates adulteration. Prepare two different types of microscopic sections of herbs: transverse sections of leaf, stem, root and bark; longitudinal sections of stem and root including their barks in Microscopic Sections of the Herb (2.6.7.1).

b) Powder Study of Processed herbs

The study of powders are gaining importance in Pharmacopoeias as it is simple, quick and needs very little of the herb to find tissues of diagnostic value. Prepare the powders as described in Preparation of Powders (2.6.7.2).

Powder study includes. Organoleptic evaluation, Fluorescence characteristics and Microscopic features.

Organoleptic evaluation

Observe colour, taste, odour and form of the processed herb.

Colour. green (leaves and aerial part); brown (barks & underground organs); specific color (specific herb).

Odour. Do not inhale the powder direct. Rub a little on the palm remove the powder from the palm and smell. Characteristic odour may give identification, specially volatile oil containing drugs.

Taste. Put 1-2 drops of water in a small watch glass. Dip a needle in it and dip again in the powder. Mix well with the needle. Dip the little finger in the water extract of the powder and taste. It is necessary to wash the mouth with water before and after tasting the powder, may give an idea about alkaloid containing herbs and bitters.

Form. Feel the powder between thumb and index finger. Describe as smooth, gritty particles and mucilaginous.

Caution. Do not taste unknown herbs.

Fluorescence characteristics

Observe the Fluorescence characteristics (2.6.7.3).

Microscopic features

Preparation of slides

Add 5 ml of *chloral hydrate* solution and 2-3 drops of *hydrochloric acid* to 200 mg of processed herb in a small test tube and place the test tube in a beaker and heat it on a water bath for 15 minutes or till it become transparent. Cool and allow the solution to settle or centrifuge the solution. Decant or filter or pipette out the liquid into another test tube. Keep it aside to perform tests. Use the residue after diluting with *chloral hydrate* and spread on a slide so as to give not more than 10-12 elements in a field. Take 1-2 drops on a slide, add a drop of *phloroglucinol* and allow it to evaporate followed by a drop of *concentrated hydrochloric acid*. To avoid air bubbles while placing the cover glass, hold the slide in the left hand and the cover glass with the right hand. Tilt the slide slightly to the left, while placing the edge of the cover glass, to form a line of the liquid along the edge and slowly drop the cover glass. Remove the excess liquid from the slide with blotting paper without applying pressure.

Prepare at least 3-4 slides for powder study. All the tissues may not be present in one slide. The slide should not contain more than 10-12 elements. Dilute if necessary.

Study of the prepared slides

In powders, microscopic feature are scattered as single cells, tissues and fragments of cells. They are seen in surface view (S.V), transverse view (T.V) and longitudinal view (L.V). Hence a thorough knowledge of tissues in general is an important

Table 1: Identification based on trichomes, starch grains and crystals for 10 common herbs

Drug	Trichomes	Starch	Crystals	Specific tissues
Senna	+	–	+	Paracytic stomata, warty, unicellular, conical trichomes, cluster crystals, crystal sheath
Digitalis	+	–	–	Stomata, typical collapsed trichomes
Cinnamon	–	+	+	Typical sclereids, fibres, microcrystals
Cinchona	–	+	+	Llarge fibres, idioblasts of micro- crystals
Ipecac	–	+	+	Raphides, tracheids, cork
Liquorice	–	+	+	Numerous starch, crystal sheath,large vessels cork
Fennel	–	-	+	Small rosette crystal in endosperm, reticulate, parenchyma, vittae
Nux-vomica	+	–	–	Typical lignified trichomes with sclereid bases, endosperm thick cellulosic wall
Clove	–	–	+	Pollen, numerous cluster crystals, typical oil glands
Quassia	–	+	+	Few starch, fibres, large vessels

requirement before the study. This will help in identification of morphological part, based on starch, trichomes, crystals as shown in Table-1, thereby confirm the identification of herb with reference to specific characters followed by micro-chemical/chemical tests and study the slide under low power (10 × 10 magnification) from left to right and right to left. List out the general and specific (diagnostic) characters.

c) Quantitative microscopy of herbs

A quantitative microscopy of herbs is done by counting a specific histological features, leaf constants: stomatal number and stomatal index, vein-islet and veinlet termination number and palisade ratio. These features are compared with the standard samples.

Stomatal number and Stomatal index

Stomatal number is the average number of stomata per square mm of the epidermal surface of the leaf. This is also known as stomatal frequency, is variable and depends on certain factors like age, growth and climatic zones, but the ratio of the stomatal number of the upper surface to the lower surface is constant in a species. It is determined by counting the stomata in a square mm. Determine *Stomatal number* for both upper and lower epidermis. These may also be determined on larger pieces of epidermal tissue in powders.

Stomatal index (S.I) is the percentage of epidermal cells modified into stomata. It is fairly constant and is independent of the factors like the age, growth and climatic zones. The stomata and the epidermal cells in a unit area are counted by microscopic methods. Determine the Stomatal indices for both upper and lower epidermis.

Palisade ratio

Palisade cells bear a definite relationship to the epidermal cells, a diagnostic feature in the identification of leaves. It is defined as the number of palisade cells under each epidermal cell. This is determined by tracing a group of four or five epidermal cells and calculating the average number of palisade cells for one epidermal cell. A total of 100 epidermal cells are taken for calculations, 4 epidermal cells of 25 readings or 5 epidermal cells of 20 readings to get the count of 100 cells. This may be carried out on leaf pieces. Determine upper epidermis for dorsiventral leaf; upper and lower surfaces in isobilateral leaves. Use either 5 × 40 or 10 × 40 magnification depending on the cell size.

Vein-islet number. Vein-islet number is defined as the number of vein-islets per sq mm of the leaf surface, midway between the midrib and the margin. The leaf sample, after soaking in water is treated successively with *sodium hypochlorite* and 10 per cent *hydrochloric acid* to remove *calcium oxalate* and finally *chloral hydrate*. The camera lucida is set- up and by means of a stage micrometer the paper is divided into squares of one square millimeter. In the cleared preparation veins are traced in four continuous squares, in a square of 2 mm × 2 mm. Each vein-islet is numbered during counting. The range and average is determined in 10 sets of 2 mm × 2 mm area. Observe under 10 × (eye piece) and 4 × (objective).

Vein-islet determination number. It is defined as the number of veinlet terminations per sq mm of the leaf surface. It is the

ultimate free termination of a veinlet or branch of a veinlet. It is counted in the same preparation as for vein-islet number. The total number of vein-islets and veinlet terminations in four adjoining squares are divided by four in order to get the value in one square mm. The range and average is determined in 10 sets of 2 mm × 2 mm area. Observe under 10 × (eye piece) and 4 × (objective) (2.6.7.4).

d) Quantitative microanalysis of processed herbs

Foreign Organic Matter is determined by Lycopodium spore method.

Lycopodium spore method. Lycopodium spores are from club moss, *Lycopodium clavatum* Linn. (Fam. Lycopdodiaceae). The spores are yellow in colour, spheroidal tetrahedral in shape with reticulate surface. They have uniform average diameter of 25 microns. one milligram contains average of 94000 spores. They have uniform moisture content hence the weight remains the same and are resistant to pressure. That is why these spores are used to evaluate powdered drug by comparison.

Limitations

i. First the presence of foreign matter has to be established.

ii. Quantitative values for the pure sample need to be determined.

iii. The drug should possess characteristic identifiable particle and distinct from others and resistant to chemical treatment as below:

a) Discrete particle uniform in size and shape like starch granules, crystals, etc. In this the weight of particle is proportional to the number.

b) Particles composed of a layer of cells, one cell thick, same type of cells, like epidermis, layer of sclerenchymatous cells etc. In this the weight of particle is proportional to the sum of their areas.

c) Particles having rod-like shape, the length fairly uniform thickness like ribs of trichomes (*nuxvomica*). In this the weight of particle is proportional to the sum of their lengths.

Determination of foreign organic matter by lycopodium spore method refered through Determination of Foreign Organic Matter by Lycopodium spore method (2.6.7.5).

e) Microchemistry of herbs and processed herbs

Microchemistry. It is the specific tests carried out on sections of the herbs/micro-quantities of powder/drops of extracts with required reagents on slides. Colour change or precipitate formation or crystal formation is observed with naked eye or with the help of microscope.

Micro-sublimation. Some powders give sublimates on heating like anthraquinones. Take 50 mg of powder in a watch glass. Place this on a warm hot plate and immediately place a micro-slide on top of the watch glass. A sublimate is formed on the lower surface of the slide.

Micro- extraction and micro-filtration. Take a small quantity of the powder herb on a micro-slide. Mix it with a drop of solvent and place a cover-glass. Leave it aside for two minutes. Take a filter paper strip cut a triangle with the base equal to the width of the cover-glass (18 mm) and the two other sides drawn into tapering cone 4 cm long. Soak this in solvent and drain excess. Place the filter paper touching the right hand side edge cover glass and hold the micro-slide in a slant position above another micro-slide. Add solvent drop by drop to the drug under cover-glass from the left side. The solvent passes through the drug and extracts the constituents and passes through the filter paper by capillary attraction. It filters through and collects as drops on the slide kept below. Discard the first colourless drops and collect the drops of extract on different slides (3-4) for testing or a small test tube.

Clarification of microscopic particles

The presence of certain cell contents, such as starch grains, aleurone grains, plastids, fats and oils, may render sections non-translucent and obscure certain characteristics. Reagents that dissolve some of these contents can be used in order to make the remaining parts stand out clearly or produce a penetrating effect. This renders the section more transparent and reveals details of the structures.

If the refractive index of the clarifying agent is close to that of the cell structure, the material being tested becomes almost invisible; if it differs appreciably, the material becomes markedly evident.

The most frequently used clarifying agents are described below:

Chloral hydrate. On gentle heating *chloral hydrate* dissolves starch grains, aleurone grains, plastids, volatile oils, expand collapsed and delicate tissue without causing any undue swelling of cell walls or distortion of the tissues.

It has a refractive index of 1.44 -1.48. It is the best reagent for rendering *calcium oxalate* clearly evident and is particularly useful for small crystals. However, when allowed to stand, it slowly dissolves *calcium oxalate*, owing to an increase in acidity.

Lactochloral. This has a similar use to *chloral hydrate*, is usually applied to sections that are difficult to clarify. It may be used cold. Before use, any air present in the specimen should be removed by placing in a dessicator and applying a vacuum.

Lactophenol. It may be used cold or with heating. It has a refractive index of 1.44 and is useful for the preparation of fungi, pollen grains, most non-oily powders and parasites

such as mites and nematode worms. Sizes of starch grains can be measured accurately, but the concentric rings are usually invisible when prepared in this reagent. Crystals of *calcium oxalate* are clearly visible in *lactophenol* and shine brightly when illuminated with polarized light. This reagent dissolves *calcium carbonate* deposits with a slow effervescence, owing to the presence of *lactic acid.*

Sodium hypochlorite. This is used for bleaching deeply coloured sections. Immerse the sections in the solution for a few minutes until sufficiently bleached, wash with water and prepare the mount with *glycerol/ethanol.* The bleached sections give a negative reaction to lignin.

Solvents for fats and oils. Xylene and *light petroleum* can be used to remove fats and oils from oily powders or sections. When necessary, immerse the material in the solvent for a short time, decant the liquid and wash the material with fresh solvent.

Histochemical detection of cell walls and contents

Reagents can be applied to a powdered sample or a section on a slide by the following methods:

Add drops of the reagent to the sample and apply a cover-glass, then irrigate using a strip of filter-paper. Place drops of the reagent on the edge of the cover-glass of a prepared specimen. Place a strip of filter-paper at the opposite edge of the cover-glass to remove the fluid under the cover-glass by suction, causing the reagent to flow over the specimen.

Using the second method, the progress of the reaction may be observed under a microscope. Care should be taken to avoid using reagent or vapours that could attack the lenses or stages of the microscope.

Cellulose cell walls. Add 1-2 drops of *iodinated zinc chloride* and allow to stand for few minutes; alternatively, add 1 drop of *0.1 M iodine*, allow to stand for 1 minute, remove excess reagent with a strip of filter-paper and add 1 drop of *sulphuric acid*; cellulose cell walls are stained blue to blue-violet. On the addition of 1-2 drops of *cuoxam*, the cellulose cell walls will swell and gradually dissolve.

Lignified cell walls. Moisten the powder or section on a slide with a small volume of *phloroglucinol* and allow to stand for about 2 minutes or until almost dry. Add 1 drop of *hydrochloric acid* and apply a cover-glass; lignified cell walls are stained pink to cherry red.

Suberized or cuticular cell walls. Add 1-2 drops of *sudan red* and allow to stand for a few minutes or warm gently; suberized or cuticular cell walls are stained orange-red or red.

Aleurone grains. Add a few drops of *iodine/ethanol*; the aleurone grains will turn yellowish brown to brown. Then add a few drops of *ethanolic trinitrophenol*; the grains will turn yellow. Add about 1 ml of *mercuric nitrate* and allow dissolving; the colour of the solution turns brick red. If the specimen is oily, render it fat-free by immersing and washing it in an appropriate solvent before carrying out the test.

Calcium carbonate. Crystals or deposits of *calcium carbonate* dissolve slowly with effervescence when *acetic acid* or *hydrochloric acid* is added.

Calcium oxalate. Crystals of *calcium oxalate* are insoluble in *acetic acid* but dissolve in *hydrochloric acid* without effervescence (if applied by irrigation the acid should be more concentrated); they also dissolve in *sulphuric acid*, but needle-shaped crystals of *calcium sulphate* separate on standing after about 10 minutes. In polarized light, *calcium oxalate* crystals are birefringent. *Calcium oxalate* is best viewed after the sample has been clarified (e.g. with *chloral hydrate*).

Fats, fatty oils, volatile oils and resins. Add 1-2 drops of *sudan red* and allow to stand for a few minutes or heat gently, if necessary. The fatty substances are stained orange-red to red. Irrigate the preparation with *ethanol* and heat gently; volatile oils and resins dissolve in the solvent, while fats and fatty oils (except castor oil and croton oil) remain intact.

Hydroxyanthraquinones. Add 1 drop of *potassium hydroxide*, cells containing *1,8-dihydroxyanthraquinones* are attained red.

Inulin. Add 1 drop each of *1-naphthol* and *sulphuric acid*, spherical aggregations of crystals of inulin turn brownish red and dissolve.

Mucilage. Add 1 drop of *Chinese ink* to the dry sample, the mucilage shows up as transparent, spherically dilated fragments on a black background. Alternatively, add 1 drop of *thionine* to the dry sample, allow to stand for about 15 minutes, then wash with *ethanol*, the mucilage turns violet-red (*cellulose* and *lignified cell walls* are stained blue and bluish violet respectively)

Starch. Add a small volume of *0.02 M iodine*, a blue or reddish blue colour is produced. Alternatively, add a small volume of *glycerol/ethanol* and examine under a microscope with polarized light; birefringence is observed giving a Maltese cross effect with the arms of the cross intersecting at the hilum.

Tannin. Add 1 drop of *ferric chloride*; it turns bluish black or greenish black.

2.6.7.1. Microscopic Sections of the Herb

Preliminary treatment

Redydrate or boil the representative sample of soft tissues like leaves and flowers for 5 minutes. Soak hard and thick herbs like barks, woods, roots, rhizome and seeds usually soaked in water for 20-30 minutes or more till the tissue become soft.

Sections cutting

Cut representative pieces of the herb from the end which is softened and smoothed into suitable lengths and prepare cross or transverse sections by cutting with razor blade or microtome at a right angle to the longitudinal axis of the material. Prepare longitudinal sections by cutting in parallel with the longitudinal axis, either in a radial direction (radial section) or in a tangential direction (tangential section).

Cut the thick and soft end tissue by holding the herb in the left hand vertically between the thumb and index finger, keeping the index finger higher than the thumb to give support to the razor blade while cutting. Hold the razor blade horizontally with few a drops of water over the right hand index finger folded in and press it down with the thumb. Microtome can be used. Very small and soft tissues need support and require pith or potato cubes for cutting sections. Cut thin and even sections as possible. Place the sections immediately with a brush moistened with water to a dish containing water.

Flat, small and spherical seeds and fruits may be inserted into a notch cut into a small rubber stopper or embedded in hard paraffin (paraffin wax). Prepare a hard paraffin block, rectangular in shape, measuring about 7 × 7 × 15 mm and melt a small hole in the centre of one end using a heated needle or thin glass rod. Press the material, which should be dry or softened by exposure to moisture, into this hole. Then prepare sections with a microtome. For the examination of *mucilage*, *aleurone grains* or spherical aggregations of *inulin*, without using *water*.

Observe one of the sections under microscope. If the vessels and fibres show a round outline the section is a correct transverse section. If the vessels show spiral or reticulate thickenings and fibres as long rod like structures, the section is oblique. In case of oblique sections, place the material on a micro slide and give a cut at right angles to the axis of growth and then continue to take sections. Pick up thin transparent sections.

Preparation of specimens

Select 1-2 thin sections and transfer them to a slide with the help of a brush. Add 2 drops of *chloral hydrate* and warm for a fraction of a minute by passing the slide over 3-4 cm above the spirit lamp flame. If bunsen flame, take care not to boil. Repeat the above step till the section is clear and transparent. Cool the side and add a drop of *phloroglucinol* and allow it to evaporate, followed by a drop of concentrated *hydrochloric acid*. Add two drops of *glycerine* solution to the section. Hold the slide with the left hand slightly slanting towards left. Hold the cover glass in the right hand horizontally, with the thumb and index finger. Place one edge of the cover glass touching the liquid on the slide forming a line along the edge. Then slowly drop it on the section to avoid any air bubbles. Use blotting paper to clean the excess liquid. Do not press the cover glass. All lignified tissues take pink stain. The lignified tissues may be disintegrated as follows:

Cut the material into small pieces about 2 mm thick and 5 mm long, or into slices about 1 mm thick (tangential longitudinal sections are preferred for woods or xylem from stems).

Use one of the following methods depending on the nature of the cell walls. For tissues with lignified cell walls use either Method 1 or Method 2. For tissues where lignified cells are few or occur in small groups, use Method 3.

Method 1

Nitric acid and *potassium chlorate*. Place the material in a test-tube containing about 5 ml of *nitric acid* and heat to boiling. Add a small quantity of powdered *potassium chlorate* and allow to react, warming gently if necessary to maintain a slight effervescence; add fresh quantities of powdered *potassium chlorate* as needed. When the tissue appears to be almost completely bleached and shows a tendency to disintegrate, apply pressure with glass rod to the material. If the material breaks, interrupt the reaction by pouring the contents of the test-tube into water. Allow the material to settle, decant it and wash it with fresh water until the acidity is removed. Transfer the material onto a slide and tease it out with a needle. Add 1 drop of *glycerol/ethanol* and apply a cover-glass. The disintegrated material gives a negative reaction for lignin.

Method 2

Nitric acid and *chromic acid*. Place the material in a small dish and heat with *nitro-chromic acid* until the material breaks easily when pressure is applied with a glass rod. Wash the material repeatedly with water and transfer onto a slide. Tease out the material, add 1 drop of *glycerol/ethanol* and apply a cover-glass. The disintegrated material gives a negative reaction for lignin.

This treatment can also be carried out on a slide. Place a moderately thick section of the material on a slide, add the reagent and allow it to stand for about 20 minutes. Separate the cells by applying gentle pressure, or with a sliding movement of the cover-glass. This process is especially useful when the disintegration of the tissues of a section under the microscope needs to be observed to ascertain where isolated cells come from.

Method 3

Caustic alkali. Place the material in a test-tube containing about 5 ml of *potassium hydroxide* or *sodium hydroxide* and heat on a water-bath for 15-30 minutes until a portion breaks easily when pressure is applied with a glass rod. Decant the liquid and wash the softened material several times with fresh

quantities of water. This method is particularly useful for the disintegration of bark, seeds, leaves and flowers, faciliting the elimination of fibres, sclereids, lactiferous tissues and epidermis. The disintegrated material gives a negative reaction for lignin.

Study of sections

As per the morphological group of the herbal material, Transverse Section (T.S.), Radial Longitudal Section (R.L.S.) and Tangential Longitudinal Section (T.L.S.) are to be studied. Use 100 magnification for sections and 400 magnification for detailed study of cells in different regions, starch and pollen grains, trichome walls and pits on fibres.

2.6.7.2. Preparation of Powders

Clean the drug to be powdered. Remove all the different parts associated with the herb, even if they are officially permitted like stalks in Senna and Cloves, calyx in Capsicum. To get the correct identification of characters, cut larger pieces into smaller pieces and grind the material in an iron mortar and pestle/lab grinder. Pass through a sieve to obtain a uniform moderately fine powder (44/85). The hard drugs like woods and barks require finer powder than soft drugs like leaves and flowers. Drugs with high oil content may be treated with *petroleum ether* to remove oil, dried and the powdered. Continue powdering till all the particles pass through the desired sieve including the 'tailings'. These are usually the hard tissues and discarding these removes some of the very important characters of identification. Label with the common and botanical name of the drug. Store them in air tight bottles and keep them in a dark, cool place to prevent deterioration and colour change. Separate out small quantities for study. Never transfer back the powders once they are taken out of the containers.

2.6.7.3. Fluorescence characteristics

Take about 0.5 g each of plant powder into clean and dried test tubes. To each tube add 5 ml of different organic solvents like distilled *water, acetone, ethanol, benzene, chloroform, diethyl ether, methanol, glacial acetic acid, sulphuric acid, nitric acid, hydrochloric acid,* 5 per cent *ferric chloride,* 5 per cent *iodine, picric acid, 1 M sodium hydroxide* and *methanol* separately. Shake and allow to stand for about 20-25 minutes. Observe the solutions under the visible day light UV light, 254 nm and 366 nm for their characteristic color.

2.6.7.4. Determination of Leaf Constants

Equipment

Stage micrometer

This is a glass slide containing a calibrated scale engraved on a small square or round glass. In this 1.0 mm is divided into 10 large units of 0.1 mm each (longer lines) numbered 1 to 10. Each unit shows further division into 10 smaller units of 0.01 mm smaller lines, i.e millimicron. This is used to calibrate the eye-piece micrometer.

Eye-piece micrometer

This is an arbitrary scale of 100 divisions engraved on a thick, small, glass disc which fits into the eye-piece. The width of each eye-piece micrometer division is calibrated against the stage micrometer when required for different magnifications. These are preferably used along with a microscope mounted with a mechanical stage. Two types of apparatus are used in quantitative microscopy to trace magnified image of the object under the microscope on the paper with pencil kept at 45° angle on the working bench.

Swift-Ives Camera Lucida

This is a compact apparatus supplied in a box for careful storage. In this the image is reflected by the prisms encased in the apparatus making the image formed in the microscope and the image of paper and pencil placed on the right side of the microscope on which the image is to be drawn seen super imposed on one another. Then the image can easily be traced on the paper. This needs an adjustable drawing board to adjust the angle in order to avoid distortion while drawing and the field of view is limited and needs adjustment.

Abbe's Drawing Apparatus

In this a plane mirror is attached to a side arm which makes 45° angle to the bench surface. The light from the drawing paper, pencil and light from the image to be drawn can be seen through the eye-piece simultaneously. This does not require adjustable drawing board, gives a large field of view.

Mechanical stage

A graduated scale in centimeters on the vertical and perpendicular plane directions of the stage of microscope

Counting-field finder

In case, mechanical stage is not available, this can be used in 'Lycopodium spore method' to move the slide to different positions.

Counting Square

A pre-determined positions guide to take readings for 'Lycopodium spore method'. This prevents the repetition of counting the same fields.

Stomatal number and Stomatal index

Stomatal Index

Preparation of lamina

Take a mature leaf. If the leaf is small, the whole leaf may be taken and if the leaf is large cut 5 mm square pieces from the middle portion between the lamina and midrib.

Fresh leaf

1. Peel off the epidermis in thick leaves by breaking into pieces by sheering action. Separate epidermis and clear with it with *chloral hydrate*.
2. Cut number of 5 mm pieces from the middle portion between the lamina and midrib. Boil with *chloral hydrate* in a test tube placed on a water bath to separate the epidermis out. Carefully place it on a slide with the help of camel hair brush along with 1-2 drops of *chloral hydrate* cool and then place a cover glass.

Preparing an imprint of the epidermis

Take a little piece of *gelatin* gel (50 per cent) with the help of a needle. Smear it on a hot slide, place a fresh leaf and slightly press the leaf. Invert the slide and cool it under a tap till the gel is solidified. Then the leaf is removed. This leaves an imprint of the stomata epidermal cells.

Dry leaf

1. Heat the leaf with *chloral hydrate* in a test tube on a water-bath for 30 minutes.
2. Cut the leaf into two pieces, observe under the microscope to see whether the stomata are present on both surfaces and the epidermal cells are easy to trace.
3. Place the cleared leaf with the veins facing down. Then the upper epidermis will be visible.
4. Place the other half with veins facing upwards. Then the lower epidermis will be visible
5. Add two drops of *glycerin* and place a cover glass.
6. Label the slides as 'upper' and 'lower'.
7. Repeat the procedure with the second half, this time placing the veins side down and take out the lower epidermis.

If the leaf is too thick and dark, separate the epidermis for tracing of the cells as follows:

1. Place one half with veins facing upwards after clearing.
2. Carefully scrap off the upper tissues with the edge of a razor blade, without disturbing upper epidermis. Clean it with brush dipped in *chloral hydrate solution*. The layer of cells remaining on the slide is the upper epidermis. Turn the layer upside to trace the cells.
3. Repeat the procedure with the second half, this time placing the lower surface facing downwards.

NOTE — Usually herbs and small shrubs have stomata on both surfaces. In tree species stomata are present on the lower surface. More stomata are present on the lower surface in dorsiventral leaf, almost the same number in isobilateral leaf.

Tracing of cells

1. Draw a square of about 8-10 sq cm on a drawing sheet or any unit area.
2. Place the prepared slide on the stage of the microscope.
3. Focus epidermal cells and the stomata first with 10 × 10, followed by 10 × 40 or 10 × 20.
4. Trace the stomata and the epidermal cells in the square with the help of camera lucida and using suitable magnification
5. Trace epidermal cells and the stomata outside the square to completion on two adjacent sides of the square.
6. Number the complete epidermal cells and the stomata within the square.
7. Continue numbering the cells that are more than half on two adjacent sides of the square.
8. Calculate the Stomatal index (S.I) = number of stomata per number of stomata plus number of epidermal cells × 100

About 10 readings or count of 400 cells are taken to determine the average Stomatal Index.

Stomatal Number

Follow the procedure as given in Stomatal index

Tracing of cells

The number has to be determined per square millimeter.

1. Adjust the drawing board, if swift Camera-lucida is used. It is not necessary to adjust angle with Abbe's camera lucida.
2. Draw a line of 1 mm using 10 × 10 magnification on a drawing sheet and draw a square on that line with the help of stage micrometer.
3. Replace the stage micrometer with a prepared slide of the leaf
4. Mark the number of stomata in the square with the help of Camera-lucida
5. Count the stomata. That gives the number of stomata per sq mm.
6. Take 25 readings and calculate the average.

NOTE — Note the side, for which the stomatal number is determined. Direct counting of stomata can also be done by squared eye piece, if the drawings are not required.

Palisade ratio

Preparation of lamina

1. Take a fresh leaf/dried leaf/ powder. Cut about 5 sq mm from the lamina between the midrib and the margin of the leaf. If leaf is very small take the whole leaf.

2. Clear the leaf or powder with drop-wise addition of *chloral hydrate solution*. Warm it on a spirit lamp adding the solution from time to time till the lamina/powder is transparent. Do not boil.
3. Always keep the slide moist. Allow the slide to cool.
4. Place a cover glass. Do not place the cover glass when the slide is warm. (This causes haziness due to presence of vapour below the cover glass, making it difficult to trace).

Tracing of cells

1. Place the prepared slide on the stage of the microscope fitted with Camera-lucida. Adjust the drawing board and secure a drawing paper with drawing pins/cellotape.
2. Focus on the upper most layer position 1 of the leaf with 10 × 10 first and adjust the focus to see stomata and epidermal cells.
3. Adjust the focus with fine-adjustment screw and suitable magnification.
4. Control the light by adjusting the condenser and mirror. In bright light the cell walls are not visible.
5. Select 4 or 5 groups of epidermal cells. Avoid stomata or epidermal cell with trichome.
6. Fix the slide firmly. Do not change the position till epidermal cells and the palisade cells below them have been traced.
7. Trace 4 or 5 epidermal cells with pencil on the paper. Darken the outlines of these cells without moving the paper.
8. Using fine adjustment, lower the focus to palisade cells. Less light may be needed at this stage. Initially the cells will be seen as small rounds loosely packed. Lower the focus till the cells look bigger and compactly packed.
9. Trace the palisade cells under the 4 or 5 epidermal cells selected, but complete the palisade cell even if it falls outside the outlined epidermal cells.
10. Take 4 epidermal cells and 25 readings or 5 epidermal cells and 20 readings from different locations to get 100 cell counts for determination.
11. Remove the drawing sheet. Number the palisade cells that completely fall within the outline of the epidermal cells first, continue numbering the palisade cells which are more than half within the outline of the epidermal cells.
12. Calculate and take the minimum, average and maximum values from 25 readings

$$\text{Palisade ratio} = \frac{\text{Number of palisade cells}}{\text{Number of epidermal cells}}$$

NOTE — For isobilateral leaf make a note of the upper or lower epidermis drawn.

Vein-islet number and Vein termination number

1. Hold the leaf towards a light source and see if the vein islets are complete. If the leaf is small take the whole leaf. If the leaf is big, cut about 4 mm square from the lamina between the midrib and margin.
2. Clear the leaf by boiling with *chloral hydrate* in a test tube on a water bath till transparent.
3. Stain with *phloroglucinol* and *hydrochloric acid*. Veins take a pink stain.
4. Set up the board for drawing and fix the drawing paper with drawing pins. If using Swift Camera-lucida adjust the angle of the drawing board.
5. Draw 1 mm of stage micrometer line on the drawing sheet with 10 × 10 magnification and extend it to double the length with the help of the stage micrometer and Camera-lucida.
6. Draw a big square on this line and divide the square into four squares, two above and two below each representing 1mm of the lamina magnified to 100 times (approximately 10 sq cm).
7. Place the cleared leaf on the microscope and trace the veins and vein endings on the drawing paper.
8. Trace the veins falling beyond the lines of the squares on all sides to complete the islets.
9. Number the complete islets within the squares and then the islets that are half inside the square on two adjacent sides. The number thus obtained divided by four will give the vein islets per one square millimeter.

$$\text{Vein-islet number per square mm} = \frac{\text{Total number of vein-islets in four squares}}{4}$$

Vein termination number

In continuation to the above experiment, number the vein-endings in the above drawing in each of the vein islets. This gives the number of vein endings per four square millimeters of the lamina. Take the average per one mm.

$$\text{Vein-islet number per square mm} = \frac{\text{Total number of vein endings}}{4}$$

2.6.7.5. Determination of Foreign Organic Matter by Lycopodium spore method

A. Discrete particle uniform in size and shape

Preparation a suspending agent

Fixed oil or *suspending agent*. A mixture of 2 volumes of *glycerin*, 1 volume of *mucilage* and 2 volumes of *water*. This

keeps the spores and particles in a suspension. Dilution of the suspension should give about 10 to 20 spores in a field.

Method

1. Determine the loss on drying for the powder at 105°.
2. Mix a weighed amount of air-dry powder of the drug and a weighed amount of lycopodium spores in a small watch glass (100 mg drug and 50 mg lycopodium spores). Mix with a small flexible spatula.
3. Add oil or suspending agent. Mix for 10 minutes till a smooth paste is obtained.
4. Transfer the suspension to a small glass tube by draining with the help of a glass rod. Add more suspending agent washing down the mixture into the tube. (about 4 ml of the suspending agent is required for 50 mg of lycopodium spores). This should give about 10 to 20 spores when viewed under high power when a drop of the mixture is mounted under a cover glass.
5. Slowly oscillate the glass tube between the two palms without any air bubbles, until the suspension is uniform.
6. Take a glass tube with internal diameter of about 2-3 mm and place one drop each on four slides spread the suspension on the slide less than the area of the cover slip. Apply a coverslip and leave the slides on an even surface to settle.
7. Select 25 fields and count the spores and particles in these fields x 4.
8. Make a similar suspension as above and count particles in 25 fields.
9. Require two value to determine adulteration.
10. Calculate the percentage of Foreign Organic Matter from the formula given below

$$\text{Percentage of Foreign Organic Matter} = \frac{94000 \times 100 \times n \times w}{s \times m \times p}$$

where, m = Weight in mg of the sample, calculated on sample dried at 105°,
w = Weight in mg of the lycopodium spores;
n = Number of particles in 25 fields,
s = Number of spores in 25 fields,
p = Number of particles in per mg of the pure foreign matter dried at 105°,
94,000 = Number of spores in one mg of lycopodium.

B. Particles composed of a layer of cells one cell thick – same type of cells

Determination of Epidermal area per gram of powdered Senna leaf.

This is carried out with Senna powder as given above. But the powder is cleared with 3 ml of *chloral hydrate* by heating on a water bath. Drop of suspension should give 10 –20 spores.

1. With the help of counting field count spores present in 25 fields and trace the outlines of a epidermal particles by means of Camera-lucida using 10 × 40 magnification.
2. Cut out the tracings and weigh.
3. Cut out a 10 cm square of the same paper and find the weight.
4. Calculate the area of the tracings from the above and divide the area by 400 square (1,60,000) to get actual area of the tracings in 25 fields. Calculate the area of the air dried and correct for loss on drying.

C. Particles having rod-like shape the length fairly uniform thickness - rod like eg: ribs of trichomes (*nuxvomica*)

In this the weight of particle is proportional to the sum of their lengths. Proceed as in experiment A.

1. Weigh 0.1 g of *nuxvomica* powder (No. 90) and 0.1 g lycopodium spores.
2. Stain: Moisten with 0.5 ml *alcohol* mix thoroughly incorporate 1 ml of 1.0 per cent *safranin* in *alcohol (50 per cent)* allow to stand for 10 minutes.
3. Add 1 ml of *hydrochloric acid* to decolourise the non-lignified tissues and add sufficient suspending agent (a mixture of 7 volumes of *glycerin*, 4 volumes of *mucilage* and 5 volumes of *water*).
4. Count no. of spores in 24 fields each of two slides and trace the length of ribs in the same field by Camera-lucida at a definite magnification and measure them on the drawing sheet.
5. Divide the total length in 24 fields in cm by magnification. This gives actual length.
6. Find the weight of *nuxvomica* in 24 fields:

$$\text{Weight of nuxvomica in 24 fields} = \frac{\text{Weight of spores in 24 fields}}{94,000}$$

7. Calculate the length of ribs per mg of *nuxvomica*:

$$\text{Length of ribs per mg of nuxvomica} = \frac{\text{Actual length}}{\text{Weight of nuxvomica in 24 fields}}$$

8. Calculate the percentage of foreign organic matter.

2.6.8. Determination of Aflatoxin in Herbal Drugs

Introduction

Aflatoxins are a group of poisonous chemicals that are produced by certain species of molds that can grow on food. Aflatoxins are naturally occurring mycotoxin produced mainly

by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are common and widespread in nature and are most often found when certain grains are grown under conditions of stress such as drought. The mould occurs in soil, decaying vegetation, hay and grains undergoing microbial spoilage and invades all types of organic substrates whenever and wherever the conditions are favourable for its growth. Favourable conditions include high moisture content and high temperature. Aflatoxins are very toxic and carcinogenic.

There are four main types of aflatoxins: B_1, B_2, G_1 and G_2. In addition, there are other types of aflatoxins, namely M_1 and M_2 have been reported as contaminants of food, especially of milk. Aflatoxin B_1 is the major toxin produced.

The clinical effects of aflatoxins may include death, liver cancer, reproductive problems, anaemia, immune system suppression and jaundice.

Herbal drugs contain not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1 and AFG_2.

[*CAUTION—Aflatoxins are highly potent and extreme care should be exercised in handling aflatoxin materials. Aflatoxins are highly toxic. Handle with care. The presence of unexpected contamination with aflatoxins is to be considered in determining compliance.*]

Methods to determine aflatoxins in plant origin drugs:

Method A

The *Aflatoxin* is determined by HPTLC (2.4.17)

This HPTLC test is provided to detect the possible presence of AFB_1, AFB_2, AFG_1 and AFG_2 in any material of plant origin.

Test solution 1. Grind about 200.0 g of plant material to a fine powder. Transfer about 50.0 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 ml of a mixture of *methanol* and *water* (17:3). Shake about 30 minutes and filter. (*NOTE — if the solution has interfering plant pigments, proceed as directed for Test solution 2*). Discard the first 50 ml of the filtrate and collect the next 40 ml portion. Transfer the filtrate to a separating funnel. Add 40 ml of *sodium chloride* solution and 25 ml of *hexane* and shake for 1 minute. allow the layers to separate and transfer the lower aqueous layer to a second separating funnel. Extract the aqueous layer in the separating funnel twice, each time with 25 ml of *methylene chloride*, by shaking for 1 minute. allow the layers to separate each time, separate the lower organic layer and collect the combined organic layers in a 125-ml conical flask. Evaporate the organic solvent on a water-bath. Transfer the remaining extract to an appropriate sample tube and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for Cleanup procedure in *Test solution 2*. Otherwise, dissolve the residue obtained above in 0.2 ml of a mixture of *chloroform* and *acetonitrile* (9.8: 0.2) and shake well.

Test solution 2. Collect 100 ml of the filtrate from the start of the flow and transfer to a 250 ml beaker. Add 20 ml of *Zinc Acetate-Aluminium Chloride Reagent* and 80 ml of *water*. Stir and allow to stand for 5 minutes. Add 5.0 g of a suitable filtering aid, such as diatomaceous earth, mix and filter. Discard the first 50 ml of the filtrate and again further collect 80 ml. Prepare *Test solution 1*.

Cleanup procedure: Place a medium porosity sintered glass disk or a glass wool plug at the bottom of a 10 mm × 300 mm chromatographic tube. Prepare slurry of 2.0 g of silica gel with a mixture of *ethyl ether* and *hexane* (3:1), pour the slurry into the column and wash with 5 ml of the same solvent mixture. Allow the absorbent to settle and add to the top of the column a layer of 1.5 g of *anhydrous sodium sulphate*. Dissolve the residue obtained above in 3 ml of *methylene chloride* and transfer it to the column. Rinse the flask twice with 1 ml portions of *methylene chloride*, transfer the rinses to the column and elute at a rate not more than 1 ml/minute. Add successively to the column 3 ml of *hexane*, 3 ml of *ethyl ether* and 3 ml of *methylene chloride*; elute at a rate not more than 3 ml/minute; and discard the elutes. Add to the column 6 ml of a mixture of *methylene chloride* and *acetone* (9:1) and elute at a rate not more than 1 ml/minute. Collect this elute in a small vial and evaporate to dryness on a water-bath. Dissolve the residue in 0.2 ml of a mixture of *chloroform* and *acetonitrile* (9.8: 0.2) and shake well.

Test solution 3. If interferences still exist in the residue prepared with IAC (Immunoaffinity Column) in Test solution in Method B.

Aflatoxin solution. Dilute the *Aflatoxin RS* with *acetonitrile* in 1:5 ratio to obtain a solution having a concentration of 0.4 µg/ml each of AFB_1 and AFG_1 and 0.1 µg/ml each of AFB_2 and AFG_2.

Procedure

Determine by High Performance Thin Layer Chromatography (2.4.17), coating the plate with silica gel GF_{254}.

Mobile phase. A mixture of 85 volumes of *chloroform*, 10 volumes of *acetone* and 5 volumes of *isopropyl alcohol*.

Test solution. Separately apply 2.5, 5, 7.5 and 10 µl of the Aflatoxin solution and three 10 µl applications of either Test solution 1, Test solution 2, or Test solution 3 to a suitable thin-layer chromatographic plate coated with a of chromatographic silica gel mixture. Superimpose 5 µl of the aflatoxin solution on one of the three 10 µl applications of the Test solution. Allow the spots to dry and develop the chromatogram in an unsaturated chamber containing a solvent

system until the solvent front has moved not less than 15 cm. Remove the plate from the developing chamber, mark the solvent front and allow the plate to air-dry.

Reference solution. Aflatoxin solution.

Determination. By UV light at 366 nm.

System suitability. The four applications of the Aflatoxin solution appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the position with those of the Aflatoxin solution. Any spot obtained from the Test solution with the superimposed aflatoxin solution is not less intense than that of the corresponding aflatoxin solution.

Acceptance criteria. No spot from any of the other applications of the Test solution corresponds to any of the spots obtained from the applications of the aflatoxin solution. If any spot of aflatoxins is obtained in the Test solution, match the position of each fluorescent spot of the Test solution with those of the aflatoxin solution to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the Test solution, when compared with that of the corresponding aflatoxin in the Aflatoxin solution will give an approximate concentration of aflatoxin in the Test solution. For aflatoxins, the limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1 and AFG_2.

Method B

This HPTLC test is provided to detect the possible presence of AFB_1, AFB_2, AFG_1 and AFG_2 in any material of plant origin.

Immunoaffinity Column (IAC)

Before conditioning, adjust the IAC to room temperature. For conditioning, apply 10 ml of *Phosphate Buffered Saline* solution onto the column and let it flow through the column by gravity force at a rate of 2 to 3 ml per minute. Leave 0.5 ml of the *phosphate buffered saline* solution on top of the column until the Test solution is applied.

Test solution.

Sample extraction. Transfer about 5.0 g of a powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 ml of a mixture of 17 volumes of *methanol* and 3 volumes of *water*. Shake vigorously for not less than 30 minutes and filter. Discard the first 5 ml of the filtrate and collect the next 4 ml portion. Transfer the filtrate to a separating funnel. Add 4 ml of *Sodium chloride solution* and 2.5 ml of *hexane* and shake for 1 minute. allow the layers to separate and transfer the lower aqueous layer to a second separating funnel. Extract the aqueous layer in the separating funnel twice, each time with 2.5 ml of *methylene chloride*, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer and collect the combined organic layers. Evaporate the organic solvent on a water-bath. Transfer the remaining extract to an appropriate sample tube and evaporate to dryness on a water-bath. Cool the residue. If interferences exist in the residue, proceed as directed for cleanup procedure with IAC. Otherwise, dissolve the residue obtained above in 200 µl of *acetonitrile* and shake well.

Cleanup Procedure with IAC. The residue is dissolved in 5 ml of a mixture of 60 volumes of *methanol* and 40 volumes of *water* and then diluted with 5 ml of *water*. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 ml of *phosphate buffered saline* Solution and the elution is performed slowly with 2 ml of *methanol*. Evaporate the elute with *nitrogen* and dissolve the residue in 200 µl of *acetonitrile*.

Aflatoxin solution. Dilute quantitatively the *aflatoxins RS* with *acetonitrile* in 1:50 ratio to obtain a solution containing 0.04 µg per ml each of AFB_1 and AFG_1 and 0.01 µg per ml each of AFB_2 and AFG_2.

Analysis. Determine by HPTLC (2.4.17) coating the plate with silica gel GF_{254}.

Mobile phase. A mixture of 14 volumes of *chloroform*, 2 volumes of *acetone* and 0.03 volumes of *water*.

Test solution. Separately apply 5, 7.5 and 10 µl of Aflatoxin solution and three 10 µl applications of the test solution to a suitable HPTLC plate. Superimpose 5 µl of aflatoxin solution on one of the three 10 µl applications of the test solution. Allow the spots to dry and develop the chromatogram in a saturated chamber containing a solvent system until the solvent front has moved not less than 72 mm from the origin (80 mm from the lower edge of the plate). Remove the plate from the developing chamber, mark the solvent front and allow the plate to air-dry for 5 minutes. Compare each fluorescent spot of the test solution with those of aflatoxin solution to identify the type of aflatoxin present. The concentration of aflatoxins in the test solution can be calculated from the calibration curve obtained from the scan data with aflatoxin solution.

Reference solution. Aflatoxin solution.

Determination. By scanning fluorescence density (>400 nm) under UV light at 366 nm.

System suitability. The four applications of aflatoxin solution appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the Test solution that coincides in position with those of Aflatoxin solution. Any spot obtained from the Test solution with the superimposed aflatoxin solution is not less intense than that of the corresponding Aflatoxin solution. The mean recovery of spiked AFB_1 and AFG_2 is not less than 70 per cent.

Acceptance criteria. The limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1 and AFG_2 except when otherwise indicated.

Table 1 – Aflatoxin reference solutions

Aflatoxin reference solutions	Aflatoxins RS (µl)	Final Aflatoxin Concentration of reference solution (ng per ml)				Aflatoxin
		AFB_1	AFB_2	AFG_1	AFG_2	ΣAF
1	0	0	0	0	0	0
2	12.5	0.25	0.0625	0.25	0.0625	0.625
3	25	0.5	0.125	0.5	0.125	1.25
4	50	1	0.25	1	0.25	2.5
5	100	2	0.5	2	0.5	5
6	200	4	1	4	1	10

Method C

This test method is provided for the detection of the possible presence of AFB_1 and total aflatoxins (AF: sum of AFB_1, AFB_2, AFG_1 and AFG_2).

Reference solutions. Prepare six solutions in separate 10 ml volumetric flasks according to Table 1. Dilute with *methanol* and *water* (1:1) to volume.

Store in a refrigerator and equilibrate to room temperature before use. Always prepare fresh solution.

Immunoaffinity Column (IAC)

Use an immunoaffinity column that contains monoclonal antibodies cross reactive toward AFB_1, AFB_2, AFG_1 and AFG_2. The immunoaffinity columns have a minimum capacity of not less than 100 ng of total aflatoxin and give a recovery of not less than 80 per cent for AFB_1, AFB_2, AFG_1 and AFG_2 when 5 ng of each AFB_1, AFB_2, AFG_1 and AFG_2 is applied in 10 ml of 10 per cent *methanol* in *phosphate buffered saline* solution.

Test solution.

Extraction. Weigh 5.0 g of a sample in a centrifuge tube. Add 1.0 g of *sodium chloride* and 25 ml of a mixture of *methanol* and 0.5 per cent *sodium bicarbonate* in ratio of 7:3. Shake at 400 rpm for 10 minutes. Centrifuge for 10 min at 7000 rpm (g value = 5323 mm/s^2) or at a speed that can result in a firm pellet of residues. Immediately pipette 7 ml into a centrifuge tube, add 28 ml of *0.1 M phosphate buffer solution*, mix and filter through glass microfiber paper. Collect 25 ml of filtrate (equivalent to 1 g of test sample) into a 25 ml graduated cylinder and proceed immediately with IAC chromatography.

IAC clean up. For the clean up procedure remove the top cap from the column and connect it with the reservoir. Remove the end cap from the column and attach it to the column manifold (the fit must be tight). Let the liquid in the column pass through until the liquid is about 2 to 3 mm above the column bed. Pass 25 ml of filtrate into the reservoir.

After the column run dry. To start the flow again easily, remove the column from the manifold, add about 2 ml of *phosphate buffered saline* solution into the column, reattach the column to the reservoir and wash the column with an additional 3 ml of *phosphate buffered saline* solution and then with 5 ml of *water* (5 ml of *phosphate buffered saline* solution can be added directly to the column reservoir if other techniques are used to dislodge the air bubble at the end of the column and to start flow again easily).

The column run dry and then forces 3 ml of air through the column with a syringe. Elute with 1 ml of *methanol* and collect the analytes in a 3-ml volumetric flask. The column run dry. Stand for 1 minute, then elute with an additional 1 ml of *methanol* and collect in the same volumetric flask. Let the column run dry and force 10 ml of air through the column. Dilute the elute with *water* to volume. Use this as the Test solution and perform the analysis of aflatoxins immediately.

System suitability solution. Prepare a spiked sample by adding 5 ml of reference solution 5 to a 5 g sample and repeating the procedure for the test solution, using 20 ml instead of 25 ml of the mixture of 70 volumes of *methanol* and 30 volumes of 0.5 per cent w/v solution of *sodium bicarbonate*.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: (For post-column derivatization with PHRED cell) a mixture of 6 volumes of *water*, 25 volumes of *methanol* and 15 volumes of *acetonitrile*,
- flow rate: 0.8 ml per minute,
- detector. spectrofluorometer,
- excitation wavelength. 362 nm and fluorescence wavelength: 440 nm,
- injection volume: 50 µl.

Analysis: Post-column derivatization for aflatoxins: Inject blank (reference solution 1), reference solutions 2 to 6 and the test solution into the LC column.

Identify aflatoxin peaks in the test solution by comparing the retention times with those of reference standards. Aflatoxins elution order are AFG_2, AFG_1, AFB_2 and AFB_1. After passing through the PHRED, the AFG_1 and AFB_1 have been derivatized to form AFG_2a (derivative of AFG_1) and AFB_2a (derivative of AFB_1).

The retention times of AFG_2, AFG_2a, AFB_2 and AFB_2a are between about 14 and 27 minutes using the PHRED cell. The peaks should be baseline resolved. Construct standard curves for each aflatoxin. Determine the concentration of each aflatoxin in the test solution from the calibration curve.

NOTE—the chemical structures of the derivatives resulting from electrochemical bromination and photolysis are not the same.

Aflatoxins calibration curves. Calibration curves are prepared for each of the aflatoxins using the reference solutions containing the four aflatoxins described. These solutions cover the range of 0.25 – 4 ng per ml for AFB_1 and AFG_1 and the range of 0.0625 to 1 ng per ml for AFB_2 and AFG_2. Make the calibration curves before analysis according to Table 1 and check the plot for linearity. If the test portion area response is outside (higher) the calibration range, then the Test solution should be diluted with a mixture equal volumes of *methanol* and *water* and reinjected into the LC column.

Quantitation of aflatoxins. Quantitation of aflatoxins is performed by measuring peak areas at each aflatoxin peak retention time and comparing them with the corresponding calibration curve.

System suitability. The mean recovery of spiked AFB_1 (2 µg per kg) and the total of aflatoxins (5 µg per kg) is not less than 68 per cent and 70 per cent, respectively. The relative standard deviation (RSD) is not more than 10 per cent for AFB_1 and for the total of aflatoxins.

Calculations. Plot the peak area (response, y-axis) of each of the Aflatoxin standards against the concentration (ng per ml, x-axis) and determine the slope (s) and y-intercept (a).

Calculate the level of toxin in the sample by the following formula

$$\text{Toxin (mg/kg)} = [\{(R - a)/S\} \times V/W] \times F$$

where, R = Test solution peak area,
V = final volume of the injected Test solution (ml) which is 3 ml,
F = dilution factor. So, F = 1,
W = 1 g of test sample passed through the immunoaffinity column.

The total of aflatoxins is the sum of AFG_2, AFG_1, AFB_2 and AFB_1.

Acceptance criteria

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1 and AFG_2, except when otherwise indicated.

Method D

This test method is provided for the detection of the possible presence of AFB_1 in plant material by liquid chromatography (2.4.14).

Aflatoxins are subject to light degradation. Carry out the determination protected from day light by using UV-absorbing curtains or blinds in combination with artificial light (fluorescent tubes are acceptable). Protect aflatoxin-containing solutions from day light.

Rinse glassware before use with a 10 per cent v/v solution of *sulphuric acid* and then rinse carefully with *distilled water* until no more acid is present.

Test solution. Use an immuno affinity column containing antibodies against Aflatoxin B_1 with a capacity of not less than 100 ng of Aflatoxin B_1 and which gives a recovery of not less than 80 per cent when a solution of 5 ng of Aflatoxin B_1 in a mixture of 12.5 ml of *methanol* and 87.5 ml of *water* is passed through. Allow the immuno affinity column to reach room temperature. To 5 g of the powdered drug add 100 ml of a mixture of 30 ml of *water* and 70 ml of *methanol* and extract by sonication for 30 minutes. Filter through folded filter paper. Pipette 10.0 ml of the clear filtrate into a 150 ml conical flask. Add 70 ml of *water*. Pass 40 ml through the immuno affinity column at a flow rate of 3 ml per minute (not exceeding 5 ml per minute). Wash the column with 2 volumes, each of 10 ml, of *water* at a flow rate not exceeding 5 ml per minute and dry by applying a slight vacuum for 5-10 seconds or by passing air through the immuno affinity column by means of a syringe for 10 seconds. Apply 0.5 ml of *methanol* to the column and allow to pass through by gravity. Collect the elute in a 5 ml volumetric flask. After 1 minute, apply a second portion of 0.5 ml of *methanol*. After a further 1 minute, apply a third portion of 0.5 ml of *methanol*. Collect most of the applied elution solvent by pressing air through or applying vacuum to the column. Dilute to 5 ml with *water* and shake well. If the solution is clear, it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of aflatoxin by retention.

Aflatoxin B_1 primary stock solution: Dissolve Aflatoxin B_1 in a mixture of 2 ml of *acetonitrile* and 98 volumes of *toluene* to give a 10 µg per ml solution. To determine the exact

concentration of Aflatoxin B_1 in the stock solution, record the absorbance between 330 nm and 370 nm.

Calculate the Aflatoxin B_1 mass concentration, in micrograms per millilitre, using the following expression:

$$\frac{A \times M \times 100}{\varepsilon \times l}$$

A = Absorbance determined at the maximum of the absorption curve,
M = Molar mass of aflatoxin B_1 (312g/mol),
ε = Molar absorptivity of aflatoxin B_1 in the *toluene-acetonitrile* mixture (1930 m2/mol).

Aflatoxin B_1 secondary stock solution. Prepare a secondary stock solution containing 100 ng per ml Aflatoxin B_1 by diluting Aflatoxin B_1 primary stock solution with a mixture of 2 ml of *acetonitrile* and 98 ml of *toluene*. Wrap the flask tightly in aluminium foil and store it below 4°. Before use, do not remove the aluminium foil until the contents have reached room temperature.

Aflatoxin B_1 standard solutions. Place the volumes of aflatoxin secondary stock solution indicated in Table 2 in separate 250 ml volumetric flasks. Pass a stream of nitrogen through at room temperature until the solvent has just evaporated. To each flask, add 75 ml of *methanol*, allow the Aflatoxin B_1 to dissolve and dilute to 250 ml with *water*.

Table 2 - Aflatoxin B_1 standard solutions

Standard solution	Volume of seconday stock solution (µl)	Final concentration of standard solution (ng/ml)
1	125	0.05
2	250	0.1
3	500	0.2
4	750	0.3
5	1000	0.4

Calibration curve. Prepare the calibration curve using Aflatoxin B_1 standard solutions 1 to 5, which cover a range equivalent to 1-8 µg/kg of Aflatoxin B_1 in the herbal drug. Check the plot for linearity. If the content of Aflatoxin B_1 in the sample to be examined is outside of the calibration range, the test solution must be diluted to an aflatoxin content that should be distinguish calibration curve.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 20 volumes of *acetonitrile*, 30 volumes of *methanol* and 60 volumes of *water* (for post-column derivitization with photochemical reactor or pyridinium bromide),

B. dissolve 0.12 g of *potassium bromide* and 350 µl of *dilute nitric acid* in 1000 ml of mobile phase A (for post-column derivitization with electrochemically derived bromine),

- flow rate: 1 ml per minute,
- detector. spectrofluorometer,
- excitation wavelength. 360 nm and fluorescence wavelength: 420 nm. Recommended settings for adjustable detectors are 365 nm (excitation wavelength) and 435 nm (emission wavelength),
- injection volume: 50 µl.

Post-column derivitization with pyridinium hydro bromide perbromide (PBPB):

- pulseless pump,
- T-piece with zero dead volume,
- polytetrafluoroethylene reaction tube, 0.45 m × 0.5 mm,
- mobile phase A,
- post-column derivitization reagent: dissolve 50 mg of *pyridinium hydrobromide perbromide* in 1000 ml of *water* (store protected from light and use within 4 days),
- flow rate of the derivitization reagent: 0.4 ml per minute,
- Post-column derivatization with photochemical reactor (PHRED),
- Reactor unit with one 254 nm low pressure mercury UV bulb (minimum 8 W), polished support plate,
- knitted reactor coil, *polytetrafluoroethylene* tubing knitted tightly around the UV bulb,
- size 25 m × 0.25 mm,
- nominal void volume 1.25 ml,
- exposure time: 2 minutes,
- mobile phase A.

Post-column derivatization with electrochemically generated bromine (KOBRA).

KOBRA-cell: electrochemical cell that generates a reactive form of bromine for derivitization of aflatoxins, resulting in enhanced fluorescence

Derivation direct-current supply in series with the KOBRA-cell, providing a constant current of about 100 µA

Polytetrafluoroethylene reaction tube, 0.12 m × 0.25 mm

Mobile phase B.

Elution order are aflatoxin G_2, aflatoxin G_1, aflatoxin B_2 and aflatoxin B_1.

Calculation. calculate the calibration curve $y = ax + b$, with aflatoxin B_1 concentration (ng/ml) on the x-axis and the signal (S) on the y-axis. The aflatoxin B_1 concentration (C) in a measured solution is equal to

$$\frac{(S - b)/a}{a}$$

Calculate the aflatoxin B_1 content of the herbal drug, in nanograms per gram, using the following expression:

= mass of the herbal drug taken for analysis, in grams;

$$\frac{V_1 \times V_2 \times C}{m \times V_i}$$

where, V_1 = volume of the solvent used for extraction, in millilitres;

V_2 = final volume of solution after elution from the immunoaffinity column and dilution, in millilitres;

V_i = aliquot taken for immunoaffinity clean-up, in millilitres.

= measured aflatoxin B_1 concentration of the test solution, in nanograms per millilitre

The presence of aflatoxin B_1 may be confirmed by recording the chromatogram without post-column derivitization, which leads to a large decrease (greater than 10-fold) in the response due to aflatoxin B_1.

2.6.9. Pesticide Residue

Definition

A pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of herb.

Table 1- Permissible limits for Pesticide Residue

Sr. No.	Substance	Limit (mg/kg)
1	Alachlor	0.02
2	Aldrin and Dieldrin (sum of)	0.05
3	Azinphos-methyl	1.0
4	Bromopropylate	3.0
5	Chlordane (sum of cis-, trans – and Oxythlordane)	0.05
6	Chlorfenvinphos	0.5
7	Chlorpyrifos	0.2
8	Chlorpyrifos-methyl	0.1
9	Cypermethrin (and isomers)	1.0
10	DDT (sum of p,p-'DDT, o,p-'DDT, p,p-'DDE and p,p-'TDE	1.0
11	Deltamethrin	0.5
12	Diazinon	0.5
13	Dichlorvos	1.0
14	Dithiocarbamates (as CS2)	2.0
15	Endosulfan (sum of isomers and Endosulfan sulphate)	3.0
16	Endrin	0.05
17	Ethion	2.0
18	Fenitrothion	0.5
19	Fenvalerate	1.5
20	Fonofos	0.05
21	Heptachlor (sum of Heptachlor and Heptachlorepoxide)	0.05
22	Hexachlorobenzene	0.1
23	Hexachlorocyclohexane isomers (other than γ)	0.3 24
24	Lindane (γ-Hexachlorocyclohexane)	0.6
25	Malathion	1.0
26	Methidathion	0.2
27	Parathion	0.5
28	Parathion-methyl	0.2
29	Permethrin	1.0
30	Phosalone	0.1
31	Piperonyl butoxide	3.0
32	Pirimiphos-methyl	4.0
33	Pyrethrins	3.0
34	Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	1.0

Table 2 - Permissible Limits for Pesticide Residue banned in India (2016)

Sr. No.	Substance	Limit (mg/kg)
1	Aldrin and Dieldrin (sum of)	0.05
2	Chlordane (sum of cis-, trans- and Oxythlordane)	0.05
3	Chlorfenvinphos	0.5
4	DDT (sum of p,p-'DDT, o,p-'DDT, p,p-'DDE and p,p-'TDE	1.0
5	Diazinon	0.5
6	Endrin	0.05
7	Fenitrothion	0.5
8	Heptachlor (sum of Heptachlor and Heptachlorepoxide)	0.05
9	Lindane (γ-Hexachlorocyclohexane)	0.6

NOTE — Apart from the above, if any pesticides applied to the herb before or after harvesting should also be tested. The limit should be calculated using the following formula.

$$\frac{ADI \times M}{MDD \times 100}$$

Where, ADI = Acceptable daily intake as published by FAO-WHO, in milligrams per kilogram of body mass,
M = body mass in kilograms (60 kg),
MDD = daily dose of herbal drug, in kilograms.

If the Herb is intended for the preparation of extracts, tinctures or other pharmaceutical forms whose preparation method modifies the content of pesticides in the finished product, the limits are calculated using the following expression:

$$\frac{A \times M \times E}{MDD \times 100}$$

Where, E = Extraction factor for of the method of preparation, determined experimentally.

Higher limits can also be authorized, in exceptional cases, especially when a plant requires a particular cultivation method or has a metabolism or a structure that gives rise to a higher than normal content of pesticides.

Sampling

Method. For containers up to 1 kg, take one sample from the total content, thoroughly mixed, sufficient for the tests. For containers between 1 kg and 5 kg, take three samples, equal in volume, from the upper, middle and lower parts of the container, each being sufficient to carry out the tests. Thoroughly mix the samples and take from the mixture an amount sufficient to carry out the tests. For containers of more than 5 kg, take three samples, each of at least 250 g from the upper, middle and lower parts of the container. Thoroughly mix the samples and take from the mixture an amount sufficient to carry out the tests.

Size of sampling. If the number (n) of containers is three or fewer, take samples from each container as indicated above under Method. If the number of containers is more than three, take "$\sqrt{n}$ + 1 samples for containers as indicated under Method, rounding up to the nearest unit if necessary".

The samples are to be analyzed immediately to avoid possible degradation of the residues. If this is not possible, the samples are stored in air-tight containers suitable for food contact, at a temperature below 0°, protected from light.

Reagents. All reagents and solvents are free from any contaminants, especially pesticides that might interfere with the analysis. It is often necessary to use special quality solvents or, if this is not possible, solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be carried out.

Apparatus. Clean the apparatus and especially glassware to ensure that they are free from pesticides, for example, soak for at least 16 hours in a solution of phosphate-free detergent, rinse with large quantities of *water* and wash with *acetone* and *hexane* or *heptane*.

Test for Pesticides

The following methods may be used depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. In any case, it may be necessary to use, in addition, another column with a different polarity or another detection method (mass spectrometry) or a different method (immunochemical methods) to confirm the results obtained. This procedure is valid only for the analysis of samples of herbs containing less than 15 per cent of water. Samples with a higher content of water may be dried, provided it has been shown that the drying procedure does not affect significantly the pesticide content.

Extraction (Method-I)

To 10 g of the substance being examined, add 100 ml of *acetone* and allow to stand for 20 minutes. Add 1 ml of a solution containing 1.8 µg per ml of carbophenothion in *toluene*. Homogenise using a high-speed blender for 3 minutes. Filter and wash the filter cake with two quantities, each of 25 ml of *acetone*. Combine the filtrate and the washings and heat using a rotary evaporator at a temperature not exceeding 40° until the solvent has almost completely evaporated. To the residue add a few millilitres of *toluene* and heat again until the *acetone* is completely removed. Dissolve the residue in 8 ml of *toluene*. Filter through a membrane filter (45 µm), rinse the flask and the filter with *toluene* and dilute to 10.0 ml with the same solvent (solution A).

Purification. Examine by size-exclusion chromatography.

Chromatographic system
- a stainless steel column 30 cm × 7.8 mm packed with styrene divinyl benzene copolymer (5 µm),
- mobile phase: *toluene*,
- flow rate of 1 ml per minute.

Performance of the column. Inject 100 µl of a solution containing 0.5 g per litre of *methyl red* and 0.5 g per litre of *oracet blue* in *toluene* and proceed with the chromatography. The column is not suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary calibrate the column, using a solution containing *toluene*, at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determine which fraction of the eluate contains both insecticides.

Purification of the test solution. Inject a suitable volume of solution A (100 µl to 500 µl) and proceed with the chromatography. Collect the fraction as determined above (solution B). Organophosphorus insecticides are usually eluted between 8.8 ml and 10.9 ml. *Organochlorine* and *pyrethroid* insecticides are usually eluted between 8.5 ml and 10.3 ml.

In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduce a piece of defatted cotton and 0.5 g of silica gel treated as follows: heat silica gel for chromatography in an oven at 150° for at least 4 hours. Allow to cool and add dropwise a quantity of water corresponding to 1.5 per cent of the mass of silica gel used; shake vigorously until agglomerates have disappeared and continue shaking for 2 hours using a mechanical shaker. Condition the column using 1.5 ml of *hexane*. Prepacked columns containing about 0.50 g of a suitable silica gel may also be used, provided they are previously validated.

Concentrate solution B in a current of *helium* for chromatography or oxygen-free nitrogen almost to dryness and dilute to a suitable volume with *toluene* (200 µl to 1 ml according to the volume injected in the preparation of solution B). Transfer quantitatively onto the column and proceed with the chromatography using 1.8 ml of *toluene* as the mobile phase. Collect the eluate (solution C).

Extraction (Method-II)

To 25 g of the substance being examined, add 300 ml of *acetonitrile*: *water* (3:1) and homogenise using a high-speed blender for 5 min. Filter and wash the filter cake with two quantities, each of 25 ml of *acetonitrile* and *water* mixture. Transfer filtrate and rinse to a separating funnel.

Add 50 ml of saturated solution of *sodium chloride* and mix vigorously for 30 seconds. Add 50 ml *hexane* to the separating funnel and extract. Repeat extraction with *hexane* for another two times. Collect the *hexane* layer and pass the combined hexane layer through *sodium sulphate*. Collect the *hexane* and evaporate to dryness. Dissolve the residue in 25 ml *hexane*.

Florisil column clean up. Use florisil solid phase extraction cartridges. Using bulb pipet transfer 2 ml of the *hexane* solution containing the pesticide residue in to the florisil cartridge. Elute with 12 ml of 15 per cent *diethyl ether* in *hexane*. Further elute with 12 ml of 50 per cent *diethyl ether* in *hexane*. Collect the elutes separately and evaporate and dry using rotary evaporator. Dissolve in 0.2 ml of *n-hexane* containing 10 ng/ml of *carbophenothion* and sonicate.

Quantitative Analysis

A. Organophosphorus Insecticides or Pesticides

Examine by gas chromatography, using *carbophenothion* as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to *carbophenothion*.

Test solution. Concentrate solution B in a current of *helium* for chromatography almost to dryness and dilute to 100 µl with *toluene*.

Reference solution. Prepare at least three solutions in *toluene* containing the insecticides to be determined and *carbophenothion* at concentrations suitable for plotting a calibration curve.

Chromatographic system

- a fused silica column 30 m × 0.32 mm packed with poly (dimethyl diphenyl) siloxane (film thickness 0.25 µm),
- temperature:
  column. 80° for 1 minute, increase from 30° to 150° at a rate of 10° per minute and maintain at 150° for 3 minutes, raising the temperature at a rate of 4° per minute to 280° per minute and maintaining at this temperature for 1 minute,
- inlet port at 250° and detector at 275°,
- detection by flame ionization detector or atomic emission spectrometry detector,
- flow rate 1 ml per minute using hydrogen as carrier gas. Other gases such as helium for chromatography or nitrogen for chromatography may also be used provided the chromatography is suitably validated.,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 3.

Table 3 - Relative Retention Times of Pesticides

Sr. No.	Substance	Relative retention time
1	Dichlorvos	0.20
2	Fonofos	0.50
3	Diazinon	0.52
4	Parathion-methyl	0.59
5	Chlorpyrifos-methyl	0.60
6	Pirimiphos-methyl	0.66
7	Malathion	0.67
8	Parathion	0.69
9	Chlorpyrifos	0.70
10	Methidathion	0.78
11	Ethion	0.96
12	Carbophenothion	1.00
13	Azinphos-methyl	1.17
14	Phosalon	1.18

Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

B. Organochlorine and Pyrethroid Insecticides/or Pesticides

Examine by gas chromatography, using *carbophenothion* as the internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

Test solution. Concentrate solution C in a current of *helium* for chromatography or oxygen-free nitrogen almost to dryness and dilute to 500 µl with *toluene.*

Reference solution. Prepare at least three solutions in *toluene* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic system

- a fused silica column 30 m × 0.32 mm packed with poly (dimethyl diphenyl) siloxane (film thickness 0.25 µm),
- temperature:
  column. 80° for 1 minute, increase from 30° to 150° at a rate of 10° per minute and maintain at 150° for 3 minutes, raising the temperature at a rate of 4° per minute to 280° per minute and maintaining at this temperature for 1 minute,
- inlet port at 250° and detector at 275°,
- detection by flame ionization detector or atomic emission spectrometry detector,
- flow rate 1 ml per minute using hydrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 4.

Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

Table 4 - Relative Retention Times of Insecticides

Sr. No.	Substance	Relative retention time
1	α-Hexachlorocyclohexane	0.44
2	Hexachlorobenzene	0.45
3	β-Hexachlorocyclohexane	0.49
4	Lindane	0.49
5	δ-Hexachlorocyclohexane	0.54
6	ε-Hexachlorocyclohexane	0.56
7	Heptachlor	0.61
8	Aldrin	0.68
9	cis-Heptachlor-epoxide	0.76
10	o,p-'DDE	0.81
11	α-Endosulfan	0.82
12	Dieldrin	0.87
13	p,p-'DDE	0.87
14	o,p-'DDD	0.89
15	Endrin	0.91
16	β-Endosulfan	0.92
17	o,p-'DDT	0.95
18	Carbophenothion	1.00
19	p,p-'DDT	1.02
20	cis-Permethrin	1.29
21	trans-Permethrin	1.31
22	Cypermethrin*	1.40
23	Fenvalerate*	1.47 and 1.49
24	Deltamethrin	1.54

**The substance shows several peaks.*

2.7. TESTS ON VACCINES

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2.7.1. Composition of Polysaccharide Vaccines

O-Acetyl Groups

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume. Dilute the solution in a way that the volumes used in solution contain 30 to 600 µg of *acetylcholine* (*O-acetyl*). Take 0.3 ml, 0.5 ml and 1 ml in duplicate into six tubes (three reaction solutions and three correction solutions).

Reference solutions. Dissolve 0.150 g of *acetylcholine chloride* in 10 ml of *water* (stock solution containing 15 g of *acetylcholine chloride* per litre). Immediately before use, dilute 1 ml of the stock solution to 50 ml with *water* (working dilution 1:300 µg of *acetylcholine chloride* per ml). Immediately before use, dilute 1 ml of the stock solution to 25 ml with *water* (working dilution 2:600 µg of *acetylcholine chloride* per ml). Introduce 0.1 ml and 0.4 ml of working dilution 1 in duplicate (reaction and correction solutions) in four tubes and 0.6 ml and 1 ml of working dilution 2 in duplicate (reaction and correction solutions) in another four tubes. Prepare a blank using 1 ml of *water*.

Method. Make up the volume in each tube to 1 ml with *water*. Add 1 ml of *4M hydrochloric acid* to each of the correction tubes and to the blank. Add 2 ml of *alkaline hydroxylamine solution* to each tube. Allow the reaction to proceed for exactly 2 minutes and add 1 ml of *4M hydrochloric acid* to each of the reaction tubes. Add 1 ml of a 10.0 per cent w/v solution of *ferric chloride* in *0.1M hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (2.4.7) of each solution at 540 nm using the blank as compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the four reference solutions and the corresponding content of *acetylcholine chloride* and read from the curve the content of *acetylcholine chloride* in the test solution for each volume tested. Calculate the mean of the three values.

1 mole of *acetylcholine chloride* (181.7 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

Hexosamines

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of the container quantitatively to the flask and dilute with *water* to make up the required volume. Dilute the solution so that the volumes used in the test contain 0.125 to 0.500 mg of *glucosamine (hexosamine)*. Add 1 ml of the diluted solution into a graduated tube.

Reference solutions. Dissolve 60 mg of *D-glucosamine hydrochloride* in 100 ml of *water* (stock solution containing 0.500 g of *glucosamine* per litre). Add 0.25 ml, 0.5 ml, 0.75 ml, and 1 ml of the working dilution into four graduated tubes. Prepare a blank using 1 ml of *water*.

Method. Make up the volume in each tube to 1 ml with *water*. Add 1 ml of *8M hydrochloric acid* to each tube. Stopper the tubes and place in a water bath for 1 hour. Cool to room temperature. Add to each tube 0.05 ml of a 0.5 per cent w/v solution of *thymolphthalein* in *ethanol* (*95 per cent*); add *5M sodium hydroxide* until a blue colour is obtained and then *1M hydrochloric acid* till the solution becomes colourless. Dilute the volume to 10 ml with *water* in each tube (neutralised hydrolysates).

In a second series of 10-ml graduated tubes, place 1 ml of each neutralised hydrolysate. Add 1 ml of *acetylacetone reagent* (a mixture, prepared immediately before use, of 1 volume of *acetylacetone* and 50 volumes of a 5.3 per cent w/v solution of *anhydrous sodium carbonate*) to each tube. Stopper the tubes and place in a water bath at 90° for 45 minutes. Cool to room temperature. Add to each tube 2.5 ml of *ethanol* (*95 per cent*) and 1 ml of *dimethylaminobenzaldehyde solution* (prepared immediately before use by dissolving 0.8 g of *dimethylaminobenzaldehyde* in 15 ml of *ethanol* (*95 per cent*) and add 15 ml of *hydrochloric acid*) and dilute the volume in each tube to 10 ml with *ethanol* (*95 per cent*). Stopper the tubes, mix well by inverting and allow to stand in the dark for 90 minutes. Measure the absorbance (2.4.7) of each solution at 530 nm using the blank as compensation liquid.

Draw a calibration curve from the absorbances for the four reference solutions and the corresponding content of *hexosamine* and read from the curve the quantity of *hexosamine* in the test solution.

Methylpentoses

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume. Dilute the solution so that the volumes used in the test contain 2 to 20 µg of *rhamnose (methylpentoses)*. Add 0.25 ml, 0.5 ml and 1 ml of the diluted solution into three tubes.

Reference solutions. Dissolve 100 mg of *rhamnose* in 100 ml of *water* (stock solution containing 1 g of *methylpentose* per litre). Immediately before use, dilute 1 ml of the stock solution to 50 ml with *water* (working dilution: 20 mg of *methylpentose* per litre). Introduce 0.1 ml, 0.25 ml, 0.5 ml, 0.75 ml and 1 ml of

the working dilution into five tubes. Prepare a blank using 1 ml of *water*.

Method. Make up the volume in each tube to 1 ml with *water*. Place the tubes in iced *water* and add drop wise with continuous stirring to each tube 4.5 ml of a cooled mixture of 1 volume of *water* and 6 volumes of *sulphuric acid* to each tube. Warm the tubes to room temperature and place in a water bath. Cool to room temperature. Add to each tube 0.1 ml of a 3.0 per cent w/v solution of *cysteine hydrochloride*, prepared immediately before use. Shake well and allow to stand for 2 hours.

Measure the absorbance of each solution at 396 nm and at 430 nm (2.4.7) using the blank as compensation liquid. For each solution, calculate the difference between the absorbance measured at 396 nm and that measured at 430 nm. Draw a calibration curve from the absorbance differences for the five standard solutions and the corresponding content of *methylpentose* and read from the curve the quantity of *methylpentose* in the test solution for each volume tested. Calculate the mean of the three values.

Nucleic Acids

Test solution. Prepare a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume.

Method. Dilute the test solution, if necessary to obtain an absorbance value suitable for the instrument used. Measure the absorbance (2.4.7) at 260 nm using *water* as compensation liquid. The absorbance of a 0.1 per cent w/v solution of *nucleic acid* at 260 nm is 20.

Phosphorus

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume. Dilutions should be made in a way that the volume used in the test solution (1 ml) contains about 6 mg of *phosphorus*. Transfer 1 ml of the solution to a 10 ml ignition tube.

Reference solutions. Dissolve 0.2194 g of *potassium dihydrogen orthophosphate* in 500 ml of *water* to make a solution equivalent to 0.1 mg of *phosphorus* per ml. Dilute 5 ml of the solution to 100 ml with *water*. Transfer 0.5 ml, 1 ml and 2 ml of the dilute solutions to three ignition tubes, respectively. Prepare a blank solution using 2 ml of *water* in the same manner.

Method. To all the tubes add 0.2 ml of *sulphuric acid* (*96 per cent* w/w) and heat in an oil bath at 120° for 1 hour then at 160° until white fumes begin to appear (about 1 hour). Add 0.1 ml of *perchloric acid* and heat at 160° until the solution is decolourized (about 90 minutes). Cool and add to the tubes 4 ml, each of, *water* and *ammonium molybdate reagent* prepared by mixing 1 volume of a 25 g per litre solution of *ammonium molybdate*, 1 volume of a 100 g per litre solution of *ascorbic acid* and 1 volume of *sulphuric acid* (294.5 g per litre) and 2 volume of *water*. Heat in a water bath at 37° for 90 minutes and cool. Adjust the volume to 10 ml with *water* and observe for blue colour.

Measure the absorbance (2.4.7) at 820 nm against a blank solution. Draw a calibration curve with the absorbances of the three dilutions of the reference solutions and read from the curve for the quantity of *phosphorus* in the test solution.

Protein Content

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume. Place 1 ml of the solution in a glass tube and add 0.15 ml of a 40.0 per cent w/v solution of *trichloroacetic acid*. Shake well, and allow to stand for 15 minutes, centrifuge for 10 minutes at 5,000 rpm and discard the supernatant. Add 0.4 ml of *0.1M sodium hydroxide* to the residue obtained after centrifugation.

Reference solutions. Dissolve 0.1 g of *bovine albumin* in 100 ml of *0.1M sodium hydroxide* (stock solution containing 1 g of *protein* per liter). Dilute 1 ml of the stock solution to 20 ml with *0.1M sodium hydroxide* (working dilution 1.50 mg of *protein* per liter). Dilute 1 ml of the stock solution to 4 ml with *0.1M sodium hydroxide* (working dilution 2:250 mg of *protein* per liter). Place in six glass tubes 0.1 ml, 0.2 ml and 0.4 ml of working dilution 1 and 0.15 ml, 0.2 ml and 0.25 ml of working dilution 2. Make up the volume in each tube to 0.4 ml using *0.1M sodium hydroxide*. Prepare a blank solution using 0.4 ml of *0.1M sodium hydroxide*.

Method. Add 2 ml of *cupri-tartaric solution* prepared by adding 1 ml of solution I containing 10 g per litre of *copper sulphate* and 20 g per litre of *sodium tartrate*, to 50 ml of solution II containing 40 g per litre solution of *sodium carbonate* in *0.2 M sodium hydroxide* to each tube, shake well, and allow to stand for 10 minutes. Add to each tube 0.2 ml of a mixture of equal volumes of *phospho-molybdotungstic reagent* and *water*, solution should be prepared immediately before use. Stopper the tubes, mix to stand in the dark for 30 minutes. The blue colour is stable for 60 minutes. If necessary, centrifuge to obtain clear solutions.

Measure the absorbance (2.4.7), of each solution at 760 nm using the blank as compensation liquid. Draw a calibration

curve from the absorbances of the six standard solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

Ribose

Test solution. In a volumetric flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water*. Dilute the solution so that the volumes used in the test contain 2.5 mg to 25 mg of ribose. Introduce 0.20 to 0.40 ml of the diluted solution into tubes in triplicate.

Reference solutions. Dissolve 25 mg of *ribose* in *water* and dilute to 100.0 ml with the same solvent (stock solution containing 0.25 g per litre of *ribose*). Immediately before use, dilute 1 ml of the stock solution to 10.0 ml with *water* (working dilution: 25 mg per litre of *ribose*). Introduce 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml and 1.0 ml of the working dilution into six tubes.

Prepare a blank using 2 ml of *water*.

Make up to volume in each tube to 2 ml with *water*. Shake well and add 2 ml of a 0.5 g per litre solution of *ferric chloride* in *hydrochloric acid* to each tube. Shake, add 0.2 ml of a 100 g per litre solution of *orcinol* in *absolute ethanol*. Place the tubes in a water-bath for 20 minutes. Cool in iced *water*. Measure the absorbance (2.4.7) of each solution at 670 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbance readings for the six reference solutions and the corresponding content of *ribose* and read from the curve the quantity of *ribose* in the test solution for each volume tested. Calculate the mean of the three values.

Sialic Acid

Test solution. Transfer quantitatively the contents of one or several containers to a volumetric flask of a suitable volume so that the resulting solution will give a concentration of about 250 µg per ml of *polysaccharide* and dilute with *water* to make up the required volume. With the help of a syringe, transfer 4 ml of this solution to a 10 ml ultra filtration cell suitable for the passage of molecules of relative molecular mass less than 50,000. Rinse the syringe twice with *water* and transfer the rinsings to the ultrafiltration cell. Carry out the ultrafiltration, with constant stirring, under *nitrogen* at a pressure of about 150 kPa. Refill the cell with *water* each time the volume of liquid in it has decreased to 1 ml and continue until 200 ml has been filtered and the remaining volume in the cell is about 2 ml. Using a syringe, transfer this residual liquid to a 10 ml volumetric flask. Wash the cell with three quantities, each of 2 ml, of *water*, transfer the washings to the flask and dilute to 10.0 ml with *water* (test solution). In each of two test-tubes place 2.0 ml of the test solution.

Reference solutions. Use the reference solutions prescribed in the monograph.

Prepare two series of three test-tubes, place in the tubes of each series 0.5 ml, 1.0 ml and 1.5 ml respectively, of the reference solution corresponding to the type of vaccine under examination and adjust the volume in each tube to 2 ml with *water*. Prepare blank solutions using 2 ml of *water* in each of two test-tubes. To all the tubes add 5.0 ml of *resorcinol reagent*. Heat at 105° for 15 minutes, cool in *cold water* and transfer the tubes to a bath of iced *water*. To each tube add 5 ml of *isoamyl alcohol* and mix thoroughly. Place in the bath of iced water for 15 minutes. Centrifuge the tubes and keep them in the bath of iced *water* until the examination by absorption spectrophotometry is made. Measure the absorbance (2.4.7), of each supernatant solution at 580 nm and 450 nm using *isoamyl alcohol* as the compensation liquid. For each wavelength, calculate the absorbance as the mean of the values obtained with two identical solutions. Subtract the mean value for the blank solution from the mean values obtained for the other solutions. Draw a graph showing the difference between the absorbances at 580 to 450 nm of the reference solution as a function of the content of *N-acetylneuraminic acid* and read from the graph the quantity of *N-acetylneuraminic acid (sialic acid)* in the test solution.

Uronic Acids

Test solution. In a graduated flask of suitable volume take a preparation of a solution containing about 5 mg per ml of dry *polysaccharide*. Transfer the contents of a container quantitatively to the flask and dilute with *water* to make up the required volume. Dilute the solution so that the volumes used in the test contain 4 to 40 µg of *glucuronic acid (uronic acids)*. Add 0.25 ml, 0.5 ml and 1 ml of the diluted solution into three tubes.

Reference solutions. Dissolve 50 mg of *sodium glucuronate* in 100 ml of *water* (stock solution containing 0.4 g of *glucuronic acid* per litre). Immediately before use, dilute 5 ml of the stock solution to 50 ml with *water* (working dilution: 40 mg of *glucuronic acid* per litre). Introduce 0.1 ml, 0.25 ml, 0.5 ml, 0.75 ml and 1 ml of the working dilution into five tubes. Prepare a blank using 1 ml of *water*.

Method. Make up to volume in each tube to 1 ml with *water*. Place the tubes in iced *water* and add dropwise with continuous stirring to each tube 5 ml of *borate solution*. Stopper the tubes and place in a water bath for 15 minutes. Cool to room temperature. Add 0.2 ml of a 0.125 per cent w/v solution of *carbazole* in *absolute ethanol* to each tube. Stopper the tubes and place in a water bath for 15 minutes. Cool to room temperature. Measure the absorbance (2.4.7) of each solution at 530 nm using the blank as compensation liquid.

Draw a calibration curve from the absorbances for the five standard solutions and the corresponding content of *glucuronic acid* and read from the curve the quantity of *glucuronic acid* in the test solution for each volume tested. Calculate the mean of the three values.

2.7.2. Cell Substrates for the Production of Vaccines for Human Use

This chapter deals with various cell lines such as diploid and continuous cell lines used for the production of vaccines for human use. Testing to be carried out at various stages (cell seed, master cell bank, working cell bank, cells at or beyond the maximum population doubling level used for production).

Diploid cell line. Diploid cell line has a high but finite capacity for multiplication *in vitro*.

Continuous cell line. A continuous cell line has a capacity to multiply indefinitely *in vitro*. It may be obtained from healthy or tumoral tissue.

For injectable vaccines produced in continuous cell lines the purification process is validated to demonstrate removal of substrate-cell DNA to a level equivalent to not more than 10 ng per single human dose, unless otherwise prescribed.

Cell bank system. Production of vaccines in diploid and continuous cell lines is based on a cell bank system. The *in vitro* age of the cells is counted from the master cell banks. Each working cell bank is prepared from one or more containers of the master cell banks. Diploid cell lines such as MRC-5 and WI 38 can be used for production upto a level of 10 generations before senescence.

Cell seed. Cell seed shall have the information on source, history and characterization. For characterization of the cell seed the properties such as the identity of the cells (for example; isoenzymes, DNA fingerprinting), karyotype, growth characteristics, viral susceptibility and viability during storage and finite life span of the cells.

Test for extraneous agents. Cell lines for vaccine production shall be free from infectious agents. Test will depend on the origin and culture history of the cell line particularly those which are known to infect latently the species of origin such as simian virus 40 (SV40) in cell line derived from monkeys and other viruses in case of cell lines of rodent origin.

Cell lines that show the presence of infectious retroviruses are not acceptable for production of vaccines, unless otherwise justified and authorised.

Table 2.7.2.-1. – Testing of Cell Lines

Test	Cell seed	Master cell bank (MCB)	Working cell bank (WCB)	EOPC/ECB (Cells at or beyond the maximum population doubling level used for production)
1. Identity and Purity				
Morphology	+	+	+	+
Identification	+	+	+	+
Karyotype (diploid cell lines)	+	+	+(1)	+(1)
Life span (diploid cell lines)	–	+	+	–
2. Extraneous Agents				
Bacterial and fungal contamination	–	+	+	–
Mycobacteria	–	+(2)	+(2)	–
Mycoplasmas	–	+	+	–
Spiroplasmas(3)	–	+	+	–
Electron microscopy	–	+(4)	-	+(4)
Tests for extraneous agents in cell cultures (with viable cells or equivalent cell lysate)	–	+	+	+
Tests in suckling mice and eggs	–	–	+(5)	+(5)
Test for specific viruses by NAT	–	+(6)	+(6)	+(6)
Test for viruses using broad molecular methods	+(7)	+(7)	+(7)	+(7)
Retroviruses	–	+(4)	–	+(4)
3. Tumorigenicity				
Tumorigenicity	+(8,9)	–	–	+(8)

(1) The diploid character is established for each WCB but using cells at or beyond the maximum population doubling level used for production.

(2) If the cells are susceptible to infection with Mycobacterium tuberculosis or other species.

(3) If insect cells or raw materials of plant origin are used.

(4) Testing is carried out for the MCB, but using cells at or beyond the maximum population doubling level used for production.

(5) Testing is carried out for each WCB, but using cells at or beyond the maximum population doubling level used for production.

(6) Specific tests for possible contaminants (e.g. viruses) defined according to a risk assessment based on the origin of the cells and on the potential extraneous agents inadvertently introduced during production processes or through the use of animal or plant derived raw materials. The appropriate testing stages should be selected based on the risk assessment.

(7) These methods may be used either as alternative to in vivo tests and specific NAT or as supplement or alternative to in vitro culture tests based on the risk assessment and in agreement with the competent authority. The appropriate testing stages should be selected based on the risk assessment.

(8) The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and they do not need to be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic, for example CHO and BHK-21.

(9) Testing is carried out on the cell seed, but using cells at or beyond the maximum population doubling level used for production.

Tumorigenicity. For the preparation of live vaccines, cell line must not be tumorogenic at any population doubling level used for vaccine production. Tumorogenicity test is carried out by *in vivo* test in one of the animal systems such as athymic mice (Nu/Nu genotype), new born mice, rats or hamsters that have been treated with anti-thymocyte serum or globulin, thymectomised and irradiated mice that have been reconstituted (T^-, B^+) bone marrow from healthy mice.

MRC-5, the WI-38 and the FRhL-2 cells lines are recognized as being non-tumorogenic and further testing is not necessary.

Chromosomal characterization. Comply with the test for extraneous agents (2.7.3) in cell culture. The cells are of Syrian origin they are also inoculated into rabbit kidney cell cultures to test for herpes virus B (*Cercopithecid herpes virus1*).

Retroviruses. Examine for the presence of retroviruses using one of the tests such as infectivity assays, transmission electronic microscopy or reverse transcriptase assays.

Tests in animals. Inject intramuscularly (or for suckling mice, intraperitoneally or by deep subcutaneous route) into each of the following groups of animals atleast 10^7 viable cells divided equally between the animals in each group:

(a) two litters of suckling mice less than 24 hours old, comprising no less than 10 animals,

(b) ten adult mice,

(c) five guinea-pigs,

(d) five rabbits.

Observe the animals for at least 4 weeks. Investigate animals that become sick or show any abnormality to establish the cause of illness. No evidence of any extraneous agent is found. The test is not valid unless at least 80 per cent of the animals in each group remain healthy and survive to the end of the observation period.

Cells obtained from rodent species (for example Chinese hamster ovary cells or baby hamster kidney cells) tests for antibodies against viral contaminants of the species (rodent) are carried out on animals that have received injection of the cells.

Tests in eggs (only required for avian cell substrates). Using an inoculum of 10^6 viable cells per egg, inoculate the cells into the allantoic cavitiy of 10 Specified Pathogen (SPF) embryonated eggs (2.7.7) 9 to 11 day old and into the yolk sac of 10 SPF embryonated hen's eggs, 5 to 6 days old. Incubate for not less than 5 days. Test the allantoic fluids for five days for the presence of haemagglutinins using mammalian and avian red blood cells. No evidence of extraneous agent is found. The test is not valid if less than 80 per cent of the embryos remain healthy and alive till the end of observation period.

Chromosomal monitoring. Examine at least 500 cells in metaphase at the production level or at any passage thereafter. Carry out examination for the same features and in the same manners as described above. Only cell banks that have normal karyotype are used for vaccine production.

Sterility (2.2.11). Complies with the test for sterility using 10 ml sample.

Mycoplasmas (2.7.4). Complies with the test for absence of mycoplasmas using 10 ml sample.

Test for extraneous agents in cell cultures (2.7.3). Complies with the test for extraneous agents in cell cultures.

Mycobacteria (2.7.14). Carry out the test for presence of Mycobacterium spp. By culture methods known to be sensitive for the detection of these organisms using 5 ml sample.

Spiroplasmas. Spiroplasmas may be introduced into cell substrates through contamination of raw materials of plant origin or when insect cell lines are used. When appropriate,

the MCB and each WCB are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (2.6.7) may be used to detect spiroplasmas after validation and agreement from the competent authority. Use one or more containers for the test.

Electron microscopy. The MCB is examined by electron microscopy for the presence of extraneous agents. Cell lines are maintained at the temperature routinely used for production and taken at or beyond the maximum population doubling level used for production. In addition, insect cell lines are maintained at temperatures above and below those routinely used for production and may also be subjected to other treatments such as exposure to chemical stressors. For insect cell lines the maintenance temperatures and treatments used are agreed with the competent authority, along with the number of sectioned cells to be examined.

2.7.3. Extraneous Agents in Viral Vaccines

In those tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs comply with the requirements prescribed above under chicken flocks free from specified pathogens for the production and quality control of vaccines.

Virus seed lot. Take samples of the virus seed lot at the time of harvesting and, if not tested immediately, keep them at a temperature below -60°.

Adult mice. Inoculate each of at least 10 adult mice, each weighing between 15 to 20 g, intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the virus seed lot. Observe the mice for at least 21 days. Carry out autopsy of all mice that die after the first 24 hours of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice which are observed for 21 days. The virus seed lot complies with the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Suckling mice. Inoculate each of at least 20 mice, less than 24 hours old, intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the virus seed lot. Observe the mice daily for at least 14 days. Carry out autopsy of all mice that die after the first 24 hours of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice which are observed daily for 14 days. The virus seed lot passes the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80.0 per cent of the original inoculated mice survive the observation period.

Guinea-pigs. Inoculate intraperitoneally into each of at least five guinea-pigs, each weighing between 350 to 450 g, 5 ml of the virus seed lot. Observe the animals for at least 42 days for signs of disease. Carry out autopsy of all guinea-pigs that die after the first 24 hours of the test, or that show signs of illness and examine macroscopically; examine the tissues both microscopically and culturally for evidence of infection. sacrifice the animals that survive the observation period and examine in a similar manner. The virus seed lot passes the test if no guinea-pig shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80.0 per cent of the guinea-pigs survive the observation period.

Virus seed lot and virus harvest. Take samples at the time of harvesting and, if not tested immediately, keep them at a temperature below -60°.

Sterility (2.2.11). Complies with the test for sterility using 10 ml sample.

Mycobacteria (2.7.14). Carry out the test for the presence of *Mycobacterium spp.* by culture methods known to be sensitive for the detection of these organisms using 5 ml sample.

Other extraneous agents (*test in cell culture*). Samples equivalent, unless otherwise prescribed, to 500 doses of vaccine or 50 ml, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures. If the virus is grown in human diploid cells, the neutralised virus harvest is also tested on a separate culture of the diploid cells. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, from a separate culture, are also inoculated. The cells are incubated at 35° to 37° and observed for a period of 14 days. The virus seed lot or harvest passes the tests if none of the cell cultures shows evidence of the presence of any extraneous agents not attributable to accidental contamination. The test is not valid unless at least 80 per cent of the cell cultures remain available for observation during the observation period.

Avian viruses (*required only for virus propagated in avian tissues*). Neutralise a sample equivalent to 100 doses or 10 ml, whichever is the greater. Using 0.5 ml per egg, inoculate a group of fertilised SPF eggs, 9 to 11 days old, by the allantoic route and a second group, 5 to 7 days old, into the yolk sac.

Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes, examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

Production cell culture: Control cells

Examine the control cells microscopically for freedom from any virus causing cytopathic degeneration throughout the time of incubation of the inoculated production cell cultures or for not less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the tests described below.

Haemadsorbing viruses. Examine not less than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea pig red blood cells. If the guinea pig red blood cells have been stored, they shall have been stored at 2° to 8° for not more than 7 days. Read half of the cultures after incubation at 2° to 8° for 30 minutes and the other half after incubation at 20° to 25° for 30 minutes. No evidence of haemadsorbing agents is found.

Other extraneous agents (*tests in cell cultures*). Pool the supernatant fluids from the control cells and examine for the presence of extraneous agents by inoculation of simian kidney and human cell cultures. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, but from a separate culture, are also inoculated. In each cell system, at least 5 ml is tested. Incubate the inoculated cultures at a temperature of 35° to 37° and observe for a period of 14 days. No evidence of extraneous agents is found.

Avian leucosis viruses (*required only if the virus is propagated in avian tissues*). Carry out a test for avian leucosis viruses on the supernatant fluid from the control cells.

Control eggs

Haemagglutinating agents. Examine 0.25 ml of the allantoic fluid from each egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows. Inoculate a 5 ml sample of the pooled amniotic fluids from the control eggs in 0.5 ml volume into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

Avian leucosis viruses. Use a 10 ml sample of the pooled amniotic fluids from the control eggs. Carry out amplification by five passages in leucosis-free, chick-embryo cell cultures; carry out a test for avian leucosis using cells from the fifth passage. The control eggs comply with the test if no evidence of the presence of avian leucosis viruses is found.

Other extraneous agents. Inoculate 5 ml samples of the pooled amniotic fluids from the control eggs into human and simian cell cultures. Observe the cell cultures for 14 days. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

2.7.4. Test for Absence of Mycoplasmas

Use method A or method B or any other suitable validated method approved by National Regulatory Authority.

Method A

The test for mycoplasma is prescribed for master cell bank, working cell bank, virus seed lots, control cells, virus harvests, bulk vaccine and for the final lot (batch). The methods shall be used as described below.

Master Cell Bank	Standard culture method and/or indicator cell culture method
Working Cell Bank	Standard culture method and/or indicator cell culture method
Virus Seed Lots	Standard culture method and/or indicator cell culture method
Control Cells	Standard culture method and/or indicator cell culture method
Virus Harvest	Standard culture method
Bulk Vaccine	Standard culture method
Final Lot	Standard culture method

Where the test for mycoplasmas is prescribed for a virus harvest, bulk vaccine or for final lot (batch), indicator cell culture method may also be used, where necessary for screening of media.

Standard culture method

Culture media. The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the stated incubation conditions of small numbers of mycoplasmas that may be present in the preparation under examination. Liquid media must contain *phenol red dye.* The range of media chosen is shown to have satisfactory nutritive properties for at least the microorganisms described below. The nutritive properties of each new batch of medium are verified for the appropriate microorganisms in the list. When testing for mycoplasmas in

the product to be examined, at least one of the following species will be included as a positive control:

Achdeplasma laidlawii (vaccines for human and veterinary use where an antibiotic has been used during production).

Mycoplasma gallisepticum (where avian material has been used during production of vaccine or where the vaccine is intended to be used in poultry).

Mycoplasma hyorhinis (non-avian veterinary vaccines)

Mycoplasma synoviae (where avian material has been used during production of vaccine or where the vaccine is intended to be used in poultry).

Mycoplasma orale (for vaccines of human and veterinary use).

Mycoplasma pneumoniae or other suitable species of D-glucose fermenter such as *Mycoplasma fermentans* (for vaccines of human use).

The test strains are field isolates having undergone not more than fifteen subcultures and are stored frozen (-20° or lower) or freeze-dried. After cloning, the strains are identified as being of the required species by a suitable method, and are compared with type cultures, for example.

A. laidlawii	NCTC	10116	CIP 75.27	ATCC 23206
M. gallisepticum	NCTC	10115	CIP 104967	ATCC 19610
M. orale	NCTC	10112	CIP 104969	ATCC 23714
M. pneumoniae	NCTC	10119	CIP 103766	ATCC 15531
M. synoviae	NCTC	10124	CIP 104970	ATCC 25204
M. fermentans	NCTC	10117	CIP 105680	ATCC 19989
M. hyorhinis	NCTC	10130	CIP 104968	ATCC 17981

Incubation condition. Incubate liquid media in tightly stoppered containers at 35° to 38°. Incubate solid media in microaerophilic conditions (*nitrogen* containing 5 to 10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface at 35° to 38°.

Test for nutritive properties. Each new batch of medium must be subjected for this test by inoculating the chosen media with suitable number of test microorganisms as described below.

60 mm plate containing 9 ml of media	Not more than 100 colony forming units.
100 ml of liquid media.	Not more than 100 colony forming units.

Use separate plate and containers for each species of microorganisms and incubate the media. Make subcultures from 0.2 ml of liquid medium to solid medium at specified intervals as mentioned under test for mycoplasmas. The solid medium complies with the test if adequate growth is found for each test micro-organism (growth obtained does not differ by a factor greater than 5 from the value calculated with respect to the inoculum). The liquid medium complies with the test if growth on agar plates sub cultured from the broth is found for at least 1 subculture for each test microorganism.

Inhibitory substances. The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of mycoplasmas.

Carry out the test for nutritive properties in the presence and absence of the preparation under examination. If growth of the test micro-organisms occurs more than one subculture sooner in the absence of the product to be examined than in its presence, or if plates directly inoculated with the product to be examined have fewer than 1/5 of the number of colonies of those inoculated without the product to be examined, inhibitory substances are present and they must be neutralized or their effect otherwise countered, for example by passage in substrates not containing inhibitors or dilution in a larger volume of medium before test. If dilution is used, larger medium volumes may be used or the inoculum volume may be divided among several 100 ml flasks. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization.

Test for mycoplasmas. Carry out the test for *Mycoplasma* in the preparation under examination by inoculating the appropriate volume of the preparation as described below.

Media	Inoculum
60 mm plate containing 9 ml of media	0.2 ml
100 ml of liquid media	10 ml

If pH change occurs significantly on the addition of the preparation in liquid medium restore the original pH of the liquid medium by the addition of a solution of *sodium hydroxide* or *hydrochloric acid.*

Inoculate at least 2 plates of solid medium and at least 2 tubes of liquid media for the preparation under examination. Incubate one plate and tube aerobically and second plate and tube anaerobically. Incubate liquid media for 20 to 21 days. Incubate solid media for not less than 14 days, except those corresponding to the 20 to 21 day subculture, which are incubated for 7 days. At the same time, incubate an uninoculated 100 ml portion of liquid medium and agar plates as a negative control.

At the same time, incubate an uninoculated 100 ml portion of liquid medium and agar plates to serve as control. Subculture each liquid medium on 2nd to 4th day after inoculation, by inoculating atleast one plates of each solid medium with

0.2 ml. Repeat the procedure between 6th and 8th days again between 13th and 15th days and again between 19th and 21st days of the test. Observe the liquid media every 2 or 3 days and if there is any change in colour, subculture immediately. Observe solid media once per week.

If the liquid media show bacterial or fungal contamination, the test is invalid. The test is valid if atleast one plate per medium and per inoculation day can be read. Include in the test positive controls produced by inoculating not more than 100 colony forming units of atleast one test microorganism on agar medium or into broth medium. Where the test for mycoplasmas is carried out regularly and where possible, it is recommended to use the test micro-organisms in regular rotation. The test micro-organisms used are those listed under culture media.

At the end of the prescribed incubation period, examine all the inoculated solid media microscopically for the presence of mycoplasma. The product passes the test if the growth of mycoplasmas colonies has not occurred in any of the inoculated media. If growth of mycoplasmas has occurred, the test may be repeated once using twice the amount of inoculum, media and plates. The product does not comply with the test if growth of typical mycoplasmas colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of mycoplasmas on atleast 1 subculture plate. The test is invalid if one or more of the negative controls show growth of mycoplasmas. If suspect colonies are observed, a suitable validated method may be used to determine whether they are due to mycoplasmas.

Indicator cell culture method

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from mycoplasmas.

If for viral suspension the interpretation of results is affected by marked cytopathic effects, the virus may be neutralized using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

Verification of the substrate

Use Vero cells or another cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 CFU (colony-forming units) or CFU-like micro-organisms of suitable reference strains of *Mycoplasma orale* strain (NTCC 10112 CIP 104969 ATCC 23714) and *Mycoplasma hyorhinis* (ATCC-29052) or any other suitable strain.

The cells are suitable if both reference strains (or strain used) are detected.

The indicator cells must be subcultured without an antibiotic before use in the test.

Procedure

(a) Seed culture at a suitable density (2×10^4 to 2×10^5 cells/ml, 4×10^3 to 2.5×10^4 cells/cm^2) that will yield confluence after 3 days of growth. Inoculate 1 ml of the product to be examined into the cell culture vessel and incubate at 35° - 38°.

(b) After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50 per cent confluence after 3-5 days of incubation. Complete confluence impairs visualization of mycoplasmas after staining and must be avoided.

(c) Remove the medium and rinse the indicator cells with *phosphate buffer saline pH 7.4*, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of *glacial acetic acid* and 3 volumes of *methanol* is suitable when *bisbenzimide* is used for staining).

(d) Remove the fixing solution and wash the cells with *sterile water*. Dry the slides completely if they are to be stained more than 1 hour later (particular care is needed for staining of slides after drying owing to artefacts that may be produced).

(e) Add *bisbenzimide working solution* or some other suitable DNA staining agent and allow it to stand for 10 minutes.

(f) Remove the stain and rinse the monolayer with *sterile water*.

(g) Mount each coverslip, where applicable, with a drop of a mixture of equal volumes of *glycerol* and *phosphate-citrate buffer solution pH 5.5*; blot off excess amount from the edge of the cover slip.

(h) Examine by fluorescence (330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100 to 400 x magnification or greater.

(i) Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas give pinpoints or filaments over the cytoplasm and sometimes in intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

The preparation under examination complies with the test if there is no evidence of the presence of mycoplasmas in the test cultures inoculated with it. The test is invalid if the positive controls do not show the presence of the appropriate test microorganisms.

Include in the test a negative (non-infected) control and two positive *Mycoplasma* controls, of suitable test strains. Use an Inoculum of not more than 100 CFU for the positive controls.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralized using a specific antiserum that has no inhibitory effects on *mycoplasmas* or by using a cell culture substrate that does not allow growth of the virus. The absence of inhibitory effects of serum must be demonstrated by carrying out the positive control tests in the presence and absence of the antiserum.

The following section is given for information and guidance; it does not form a mandatory part of the general method.

Special reagents for indicator cell culture method

Bisbenzimide ($C_{25}H_{27}Cl_3N_6O.5H_2O$ (Mr 624) 4-[5-[5-(4-methylpiperazin-1-yl) benzimidazol-2-yl] benzimidazol-2-yl] phenol trihydrochloridepentahydrate.

Bisbenzimide stock solution. To prepare stock solution, dissolve 5 mg of *bisbenzimide* in sterile water and dilute to 100 ml with the same solvent. Store in the dark.

Bisbenzimide working solution. To prepare working solution, dilute 10µl of *bisbenzimide* stock solution to 100 ml with *phosphate buffered saline at pH 7.4.* Use immediately.

Phosphate-citrate buffer solution, pH 5.5. To prepare buffer, mix 56.85 ml of a 2.84 per cent w/v solution of *anhydrous disodium hydrogen phosphate* and 43.15 ml of a 2.1 per cent w/v solution of *citric acid.*

Recommended media for the standard culture method

The following media are recommended. Other media may be used provided their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the preparation under examination.

I. Recommended media for the detection of *Mycoplasma gallisepticum*

(a) Liquid medium

Beef heart infusion broth (1)	90.0 ml
Horse serum (unheated)	20.0 ml
Yeast extract (250 g per litre)	10.0 ml
Thallium acetate (10 g per litre solution)	1.0 ml
Phenol red (0.6 g per litre solution)	5.0 ml
Penicillin (20,000 IU per ml)	0.25 ml
Deoxyribonucleic acid (2 g per litre solution)	1.2 ml

Adjust to pH 7.8.

(b) Solid medium

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g per litre of agar.

II. Recommended media for the detection of *Mycoplasma synoviae*

(a) Liquid medium

Beef heart infusion broth (1)	90.0 ml
Essential vitamins (2)	0.025 ml
Glucose monohydrate (500 g per litre solution)	2.0 ml
Swine serum (Inactivated at 56° for 30 minutes)	12.0 ml
β-Nicotinamide adenine dinucleotide (10 g per litre solution)	1.0 ml
Cysteine hydrochloride (10 g per litre solution	1.0 ml
Phenol red (0.6 g per litre solution)	5.0 ml
Penicillin (20,000 IU per ml)	0.25 ml

Mix the solutions of *β-nicotinamide adenine dinucleotide* and *cysteine hydrochloride and* after 10 minutes add to the other ingredients. Adjust to pH 7.8.

(b) Solid medium

Beef heart infusion broth (1)	90.0 ml
Agar purified (3)	1.4 g
Adjust to pH 7.8, sterilise by autoclaving then add. Essential vitamins (2)	0.025 ml
Glucose monohydrate (500 g per litre solution)	2.0 ml
Swine serum (unheated)	12.0 ml
β–Nicotinamide adenine dinucleotide (10 g per litre solution)	1.0 ml
Cysteine hydrochloride (10 g per litre solution)	1.0 ml
Phenol red (0.6 g per litre solution)	5.0 ml
Penicillin (20,000 IU per ml)	0.25 ml

III. Recommended media for the detection of non-avian mycoplasmas

(a) Liquid medium

Hanks' balanced salt solution (modified) (4)	800 ml
Distilled water	67 ml
Brain heart infusion (5)	135 ml
PPLO Broth (6)	248 ml
Yeast extract (170 g per litre)	60 ml
Bacitracin	250 mg
Meticillin	250 mg
Phenol red (5 g per litre)	4.5 ml
Thallium acetate (56 g per litre)	3 ml
Horse serum	165 ml
Swine serum	165 ml

Adjust to pH 7.4 to 7.45.

(b) Solid medium

Hanks' balanced salt solution (modified) (4)	200 ml
DEAE-dextran	200 mg
Agar purified (3)	15.65 mg

Mix well and sterilise by autoclaving. Cool to 100°. Add to 1740 ml of liquid medium as described above.

(1) Beef heart infusion broth

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

Sterilize by autoclaving.

(2) Essential vitamins

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavin	10 mg
Thiamine hydrochloride	100 mg
Distilled water to	1000 ml

(3) Agar purified

A highly refined agar for use in microbiology and immunology prepared by an ion-exchange procedure, which results in a preparation having superior purity, clarity and gel strength. It contains about:

Water	12.2 per cent
Ash	1.5 per cent
Acid-insoluble ash	0.2 per cent
Phosphate (calculated as P_2O_5)	0.3 per cent
Total nitrogen	0.3 per cent
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

(4) Hanks' balanced salt solution (modified)

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulphate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
Distilled water	800 ml

(5) Brain heart infusion

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water to	1000 ml

(6) PPLO broth

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

Method B. Nucleic Acid Amplification Techniques (NAT)

NAT (2.8.1) may be used for detection of mycoplasmas by amplification of nucleic acids extracted from a test sample with specific primers that reveal the presence of the target nucleic acid. NAT indicate the presence of a particular nucleic acid sequence and not necessarily the presence of the viable mycoplasmas. A number of different techniques are available. This chapter does not prescribe a particular method for the test. The procedure applied must be validated as described, taking account of the guidelines presented at the end of this section. Where a commercial kit is used, certain elements of the validation may be carried out by the manufacturer and information provided to the user.

NAT are applied where prescribed in a monograph. They may also be used instead of the culture method and the indicator cell culture method after suitable validation.

Direct NAT can be applied in the presence of cytotoxic material and where a rapid method is needed.

Cell-culture enrichment followed by NAT: the test sample and a suitable cell substrate (as described under the indicator cell-culture method) are cultured together for a suitable period; the nucleic acids are then extracted from the cells and supernatant and used for detection by NAT.

VALIDATION

Reference standards are required at various stages during validation and for use as controls during routine application of the test. The reference standards may be mycoplasmas or nucleic acids.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

- *A. laidlawii;*
- *M. fermentans*;
- *M. hyorhinis* (where cell-culture enrichment is used, a fastidious strain such as ATCC 29052 is included) ;
- *M. orale* ;
- *M. pneumoniae* or *M. gallisepticum* ;
- *M. synoviae* (where there is use of or exposure to avain material during production) ;
- *M. arginini* ;
- *Spiroplasma citri* (where there is use of or exposure to insect or plant material during production).

Demonstration of specificity requires the use of a suitable range of bacterial species other than mycoplasmas. Bacterial genera with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium, Lactobacillus* and *Streptococcus*.

Comparability studies for use of NAT as an alternative method. For each Mycoplasma test species:

- as an alternative to the culture method: the NAT test system must be shown to detect 10 CFU/ml;
- as an alternative to the indicator cell culture method: the NAT test system must be shown to detect 100 CFU/ml;

or an equivalent limit of detection in terms of the number of copies of mycoplasma nucleic acid in the test sample (using suitable reference standards of mycoplasma nucleic acid).

CONTROLS

Internal controls

Internal controls are necessary for routine verification of absence of inhibition. The internal control may contain the primer binding site, or some other suitable sequence may be used. It is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

External controls

The external positive control contains a defined number of target-sequence copies or CFUs from one or more suitable species of Mycoplasma chosen from those used during validation of the test conditions. One of the positive controls is set close to the positive cut-off point to demonstrate that the expected sensitivity is achieved. The external negative control contains no target sequence but does not necessarily represent the same matrix as the test article.

INTERPRETATION OF RESULTS

The primers used may also amplify non-mycoplasmal bacterial nucleic acid, leading to false positive results. Procedures are established at the time of validation for dealing with confirmation of positive results, where necessary.

Validation of nucleic acid amplification techniques (NAT) for the detection of mycoplasmas: guidelines

SCOPE

Nucleic acid amplification techniques (NAT) are either qualitative or quantitative tests for the presence of nucleic acid. For the detection of mycoplasma contamination of various samples such as vaccines and cell substrates, qualitative tests are adequate and may be considered to be limit tests.

These guidelines describe methods to validate qualitative nucleic acid amplification analytical procedures for assessing mycoplasma contamination. They may also be applicable for real-time NAT used as limit tests for the control of contaminants.

The two characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria).

NAT may be used as:

— a complementary test (for example, for cytotoxic viral suspensions) or for in- process control purposes;

— an alternative method to replace an official method (indicator cell culture method or culture method).

These guidelines will thus separate these two objectives by presenting first a guideline for the validation of the NAT themselves, and second, a guideline for a comparability study between NAT and official methods.

GUIDELINE FOR MYCOPLASMA NAT VALIDATION

Three parameters should be evaluated: specificity, detection limit and robustness.

Specificity. Specificity is the ability to unequivocally assess target nucleic acid in the presence of components that may be expected to be present.

The specificity of NAT is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

The ability of the NAT to detect a large panel of mycoplasma species will depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. Since NAT systems are usually based on a mix of primers, the theoretical analysis of primers and probes by comparison with databases is not recommended, because interpretation of the results may be quite complex and may not reflect the experimental results.

Moreover, as it is likely that the primers will detect other bacterial species, the potential cross-detection should be documented in the validation study. Bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium, Lactobacillus* and *Streptococcus*. However, this is not an exhaustive list and species to be tested will depend on the theoretical ability (based on primers/ probes sequences) of the NAT system to detect such other species.

Based on the results from this validation of the specificity, if a gap in the specificity of the method is identified (such as detection of non-mycoplasmal bacterial nucleic acid), an appropriate strategy must be proposed in the validation study to allow interpretation of positive results on a routine basis. For example, a second test may be performed using an alternative method without this specificity gap or using an official method.

Detection limit. The detection limit of an individual analytical procedure is the lowest amount of target nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

For establishment of the detection limit, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut- off point is the minimum number of target sequence copies per volume of sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of mycoplasmal genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

To determine the positive cut-off point, a dilution series of characterised and calibrated (either in CFUs or nucleic acid copies) in-house working strains or other international standards should be tested on different days to examine variation between test runs.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

— *A. laidlawii;*

— *M. fermentans;*

— *M. hyorhinis;*

— *M. orale;*

— *M. pneumoniae* or *M. gallisepticum*;

— *M. synoviae* (where there is use of or exposure to avian material during production);

— *M. arginini*;

— *S. citri* (where there is use of or exposure to insect or plant material during production).

For each strain, at least three independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory may test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test should be performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point. The concentration of mycoplasmas (CFUs or copies) that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to evaluate the variability of the analytical procedure.

Robustness. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

Finally, robustness of the method can be evaluated through collaborative studies.

GUIDELINE

NAT may be used instead of official methods (indicator cell culture method and/or culture method). In this case a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the alternative method and official methods. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered.

For the detection limit, acceptability criteria are defined as follows:

- if the alternative method is proposed to replace the culture method, the NAT system must be shown to detect 10 CFU/ml for each mycoplasma test species described under *Detection limit*;
- if the alternative method is proposed to replace the indicator cell culture method, the NAT system must be shown to detect 100 CFU/ml for each mycoplasma test species described under *Detection limit*.

For both cases, suitable standards calibrated for the number of nucleic acid copies and the number of CFUs may be used for establishing that these acceptability criteria are reached. The relation between CFUs and nucleic acid copies for the reference preparations should be previously established to compare the performance of the alternative NAT method with the performance of the official methods.

One of the following two strategies can be used to perform this comparability study:

- perform the NAT alternative method in parallel with the official method(s) to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains;
- compare the performance of the NAT alternative method using previously obtained data from official method validation. In this case, calibration of standards used for both validations as well as their stabilities should be documented carefully.

Comparability study reports should describe all the validation elements described in section 2 (specificity, limit of detection and variability, as well as robustness) in order to assess all the advantages and/or disadvantages of the alternative NAT method compared to official methods.

2.7.5. Test for Neurovirulence (NVT) for Live Viral Vaccines

For each test, use not less than 10 monkeys that are seronegative for the virus under test. For each monkey, inject not more than 0.5 ml of the material under examination into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be not less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a group of not less than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 hours of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if more than 20.0 per cent of the inoculated monkeys die from nonspecific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are killed show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected

clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

2.7.6. Test for Neurovirulence (NVT) for Oral Poliomyelitis Vaccine (OPV)

Monkeys used in the Neurovirulence test (NVT) comply with the requirements stated under Poliomyelitis Vaccine, Live (Oral) and weigh not less than 1.5 kg. The pathogenicity for *Macaca* or *Cercopithecus* monkeys is tested in comparison with that of a reference virus preparation for neurovirulence testing by inoculation into the lumbar region of the central nervous system after sedation with a suitable substance, for example, *ketamine hydrochloride*. A sample of serum taken before the injection shall be shown not to contain neutralising antibody at a dilution of 1.4 when tested against not more than 1,000 $CCID_{50}$ of each of the three types of poliovirus.

Number of monkeys. The vaccine and the appropriate homotypic reference virus are tested concurrently in the same group of monkeys. Equal numbers of animals are inoculated with the vaccine under examination and the reference preparation. The animals are allocated randomly to treatment groups and cages and their identity is coded so that the treatment received by each animal is concealed from the observers and the evaluators of the sections. The number of monkeys inoculated is such that in the evaluation of both the vaccine and the reference preparation not less than 11 positive monkeys are included for type 1 and type 2 virus and not less than 18 positive monkeys for type 3 virus (positive monkeys are those that show specific neuronal lesions of poliovirus in the central nervous system). More than one batch of vaccine may be tested with the same homotypic reference. Monkeys from the same quarantine group are used wherever possible, otherwise monkeys from two groups are used and equal numbers from each group are treated with the vaccine and the reference preparation. If the test is carried out on two working days, an equal number of monkeys from each group are inoculated on each day with the vaccine and the homotypic reference preparation.

Virus content. The virus contents of the vaccine and the homotypic reference preparation are adjusted so as to be as near as possible equal and between $10^{5.5}$ and $10^{6.5}$ $CCID_{50}$ per 0.1 ml.

Observation. All monkeys are observed for 17 to 22 days for signs of poliomyelitis or other virus infection. Monkeys that survive the first 24 hours but die before the 11th day after inoculation are autopsied to determine whether poliomyelitis was the cause of death. Animals that die from causes other than poliomyelitis are excluded from the evaluation. Animals that become moribund or are severely paralysed are killed and autopsied. All animals that survive until the end of the observation period are autopsied. The test is not valid if more than 20.0 per cent of the animals show intercurrent infection during the observation period.

Number of sections examined. The lumbar cord, the cervical cord, the lower and upper medulla oblongata, the midbrain, the thalamus and the motor cortex of each monkey, as a minimum, are subjected to histological examination. Sections are cut with a thickness of 15 µm and stained with *gallocyanin*. The minimum number of sections examined is as follows.

(a) 12 sections representative of the whole of the lumbar enlargement,

(b) 10 sections representative of the whole of the cervical enlargement,

(c) 2 sections from the medulla oblongata,

(d) 1 section from the pons and cerebellum,

(e) 1 section from the midbrain,

(f) 1 section from the left and the right of the thalamus,

(g) 1 section from the left and the right motor cerebral cortex.

Scoring of virus activity. For the evaluation of virus activity in the hemisections of the spinal cord and brain-stem, a score system for the severity of lesions is used, differentiating cellular infiltration and destruction of neurons as follows.

1. Cellular infiltration only (the monkey is not counted as positive),
2. Cellular infiltration with minimal neuronal damage,
3. Cellular infiltration with extensive neuronal damage,
4. Massive neuronal damage with or without cellular infiltration.

The scores are recorded on a standard form (a suitable form is shown in the requirements for Poliomyelitis Vaccine (Oral) (Requirements for biological substances No.7 World Health Organization). A monkey with neuronal lesions in the sections but that shows no needle tract is counted as positive. A monkey showing a needle tract in the sections, but no neuronal lesions is not regarded as positive. A section that shows damage from trauma but no specific virus lesions is not included in the score.

Severity scores are based on hemisection readings of the lumbar (L), cervical (C) and brain (B) histological sections. The lesion score (LS) for each positive monkey is calculated as follows.

$$LS = \frac{\left[\frac{\text{Sum of L score}}{\text{Number of hemisections}}\right] + \left[\frac{\text{Sum of C score}}{\text{Number of hemisections}}\right] + \left[\frac{\text{Sum of B score}}{\text{Number of hemisections}}\right]}{3}$$

A mean lesion score is calculated for each group of positive monkeys.

Evaluation. The comparison of the virus activity in the vaccine and the reference preparation is based on the activity in the lumbar enlargement of the cord and the degree of spread of activity from this region to the cervical enlargement and the brain. Acceptance or rejection is based on the total score of all the test animals. Individual animals showing evidence of unusually high activity, either in the lumbar region or as the result of spread from this region, are also taken into consideration in the final evaluation. The monovalent bulk passes the test if the required number of animals is positive and if none of the clinical and histopathological examinations shows a significant difference in pathogenicity between the vaccine virus and the reference material. Criteria for acceptance are given below.

Criteria. A suitable number of neurovirulence qualifying tests (for example, four tests) is carried out on each reference vaccine (types 1, 2 and 3) to provide data on the activity of such vaccines that will serve as the basis of the criteria for vaccines under test. The overall mean lesion score (M) for the replicate tests on each reference virus is calculated together with the pooled estimate of the within-test variance (s^2) and the within-test deviation (s).

Validity criteria for the results of a test on a reference preparation are established on the basis of the cumulative data from the qualifying tests. No generally applicable criteria can be given; for laboratories with limited experience, the following empirical method for setting acceptable limits for the mean lesion score for the reference preparation (X_{ref}) may be helpful.

If the mean lesion score for the vaccine under test is X_{test} and C_1, C_2 and C_3 are constants determined as described below, then :

the vaccine is not acceptable if

$$X_{test} - X_{ref} > C_1$$

the vaccine may be retested once if

$$C_1 < X_{test} - X_{ref} < C_2$$

If the vaccine is retested, the means of the lesion scores for the vaccine under test and the reference vaccines are recalculated. The vaccine is not acceptable if :

$$\frac{X_{(test1 + test2)} - X_{(ref1 + ref2)}}{2} > C_3$$

The constants C_1, C_2 and C_3 are calculated from the expressions.

$$C_1 = 2.3\sqrt{\frac{2s^2}{N_1}}$$

$$C_2 = 2.6\sqrt{\frac{2s^2}{N_1}}$$

$$C_3 = 1.6\sqrt{\frac{2s^2}{N_1}}$$

where, N_1 = number of positive monkeys per vaccine test,

N_2 = number of positive monkeys in the two tests,

2.3 = normal deviate at the 1.0 per cent level,

2.6 = normal deviate at the 0.5 per cent level,

1.6 = normal deviate at the 5.0 per cent level.

A neurovirulence test in which the mean lesion score for the reference (X_{ref}) is not compatible with previous experience is not used for assessing a test vaccine. If the test is valid, the mean lesion score for the vaccine under test (X_{test}) is calculated and compared with that of the homotypic reference vaccine.

2.7.7. Tests on Chicken Flocks free from Specified Pathogens for the Production and Quality Control of Vaccines

Where specified in a monograph, chickens, embryos or cell cultures used for the production or quality control of vaccines are derived from eggs produced by chicken flocks free from SPF. The SPF status of a flock is ensured by means of the system described below. The list of micro-organisms given is based on current knowledge and will be updated periodically.

General principles and procedures

A flock is defined as a group of birds sharing a common environment and having their own caretakers who have no contact with non-SPF flocks. Once a flock is defined, no non-SPF birds are added to it.

For SPF flocks established on a rolling basis, all replacements are hatched and reared in the controlled environment house. Subject to the agreement of the competent authorities, SPF embryos derived from a tested SPF flock from another house on the same site may be introduced. From 8 weeks of age, these replacement birds are regarded as a flock and monitored monthly in accordance with the Subsequent testing requirements. At point of lay, all these replacement birds are tested in accordance with the Initial testing requirements.

The flock is housed so as to minimize the chance of contamination. It is not set near to non-SPF flocks of birds and is housed in an isolator or on wire in a building with filtered air under positive pressure. Appropriate measures are

taken to prevent access of rodents, wild birds, insects and unauthorized people.

Personnel authorized to enter must have no contact with other birds or with agents likely to infect the flock. It is advisable for personnel to shower and change clothing or to wear protective clothing before entering the chicken house.

Items taken into the flock are sterilised. The feeds is suitably treated to avoid the introduction of undesirable micro-organisms and water is obtained from a chlorinated supply. No medication is given that could interfere with detection of disease in the flock.

A permanent record is kept of the general health of the flock and any abnormality is investigated. Factors to be monitored include morbidity, mortality, general physical condition, feed consumption, daily egg preparation and egg quality, fertility and hatchability. Dirty eggs are discarded; clean eggs may be surface-disinfected whilst warm.

The flock originates from chickens shown to be free from vertically transmitted agents. In particular, each chicken from which the flock is derived is tested repeatedly to ensure freedom from leucosis viruses and their antibodies. In order to establish the SPF status of a flock, it is kept under SPF conditions for a test period of not less than 4 months. Each bird in the entire flock is shown to be free from evidence of infection with the agents listed in Table 1 under the heading initial testing after 6 weeks and at the end of the test period.

For each new generation in an established flock, all of the birds in the flock are tested at not later than 20 weeks of age, using the tests prescribed below under Initial testing. After the initial test, monthly tests are carried out on a representative 5 per cent sample (but not less than ten and not more than two hundred birds), using the tests prescribed in Table 2 under the heading Subsequent testing, with a final test at 4 weeks after the last collection of eggs.

For all tests, blood samples are collected from an appropriate number of birds at the specified time. The resultant serum samples are examined for antibodies against the relevant agents. Serum-neutralization tests are done on pools of not

Table 1 – **Initial Testing**. Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and are of appropriate specificity.

Micro-organism	Type of test
Avian adenoviruses	Enzyme linked immunosorbent assay
Avian encephalomyelitis viruses	Enzyme linked immunosorbent assay
Avian infectious bronchitis virus	Enzyme linked immunosorbent assay
Avian infectious laryngo-tracheitis virus	Serum neutralization
Avian leucosis virus	Enzyme linked immunosorbent assay for virus and serum neutralization for antibody
Avian nephritis virus	Fluorescent antibody
Avian reoviruses	Enzyme linked immunosorbent assay
Avian reticuloendotheliosis virus	Fluorescent antibody
Chick infectious anaemia virus	Fluorescent antibody
Haemagglutinating avian adenoviruses (egg drop syndrome 76 adenoviruses; EDS 76 virus)	Haemagglutation inhibition
Infectious bursal disease virus	Serum neutralization against each serotype present in the country of origin
Influenza A virus	Enzyme linked immunosorbent assay
Marek's disease virus	Enzyme linked immunosorbent assay
Newcastle disease virus	Haemagglutination inhibition
Turkey rhinotracheitis virus	Enzyme linked immunosorbent assay
Mycoplasma gallisepticum	Agglutination, and to confirm positive test, Haemagglutination inhibition
Mycoplasma synoviae	Agglutination and, to confirm positive test, Haemagglutination inhibition
Salmonella pullorum	Agglutination

Table 2 – **Subsequent testing.** Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and are of appropriate specificity.

Micro-organism	Type of test
Avian adenoviruses	Enzyme linked immunosorbent assay
Avian encephalomyelitis viruses	Enzyme linked immunosorbent assay
Avian infectious bronchitis virus	Enzyme linked immunosorbent assay
Avian infectious laryngo-tracheitis virus	Serum neutralization
Avian leucosis virus	Enzyme linked immunosorbent assay for virus and Serum neutralization for antibody
Avian nephritis virus	Flourescent antibody
Avian reoviruses	Enzyme linked immunosorbent assay
Avian reticuloendotheliosis virus	Fluorescent antibody
Chick anaemia agent	Fluorescent antibody
Haemagglutiating avian adeno virus	Haemagglutination inhibition
Infectious bursal disease virus	Serum neutralization against each serotype present in the country of origin
Influenza A virus	Enzyme linked immunosorbent assay
Marek's disease Virus	Enzyme linked immunosorbent assay
Newcastle disease virus	Haemagglutination inhibition
Turkey rhinotracheitis virus	Enzyme linked immunosorbent assay
Mycoplasma gallisepticum	Agglutination and, to confirm positive test Haemagglutination inhibition
Mycoplasma synoviae	Agglutination and, to confirm positive test Haemagglutination inhibition
Salmonella pullorum	Agglutination

Table 3 – Monitoring for freedom from antibodies

Micro-organism	Type of test
Avian adenoviruses	Enzyme linked immunosorbent assay
Avian infectious bronchitis virus	Enzyme linked immunosorbent assay
Avian leucosis virus	Enzyme linked immunosorbent assay for virus and Serum neutralization for antibody
Avian reoviruses	Enzyme linked immunosorbent assay
Chick anaemia agent	Fluorescent antibody
Infectious bursal disease virus	Serum neutralization against each serotype present in the country of origin
Influenza A virus	Enzyme linked immunosorbent assay
Marek's disease virus	Enzyme linked immunosorbent assay
Mycoplasma gallisepticum	Agglutination and, to confirm positive test, Haemagglutination inhibition
Mycoplasma synoviae	Agglutination and, to confirm positive test Haemagglutination inhibition
Salmonella pullorum	Agglutination

more than five sera. All other tests are done on each individual serum. Positive and negative controls are used in all tests. The reagents used in the tests are standardized against international or any other validated standard reagents where these are available. For avian leucosis virus, in addition to tests for antibodies carried out on serum samples, appropriate samples are taken for testing for the virus.

In addition to serological tests, clinical examination is carried out at least once per week to verify that the birds are free from fowl-pox and signs of other infections. Necropsy and, where necessary to confirm diagnosis, histopathological examination are carried out on any bird that dies to verify that there is no sign of infection. The absence of *Salmonella* species is determined by cultural examination of faecal samples at least once every 4 weeks; a pool of up to ten samples may be used for the tests.

If a positive result is obtained in any test carried out to establish the SPF status of a flock, the flock may not be designated as an SPF flock. If a positive result is obtained in any test carried out on an established flock, the flock loses its SPF status. Special provisions apply to chick anemia virus (CAV) as described below. Any chickens, embryos or cell cultures collected since the previous negative test are not suitable for use: any preparation made from them must be discarded and any quality control tests done with them are invalid and must be repeated.

In order to regain SPF status, the flock is maintained under SPF conditions and routine 5 per cent monthly testing shall continue except that every bird in the entire flock is tested every month for infection with the particular agent that gave the positive result. Infected birds and their progeny are removed from the flock. SPF status is regained after two such consecutive tests have yielded completely negative results.

A positive result for CAV does not necessarily exclude use of material derived from the flock, but live vaccines for use in birds less than 7 days old must be produced using material from CAV-negative flocks. Inactivated vaccines for use in birds less than 7 days old may be produced using material from flocks that have not been shown to be free from CAV, provided it has been demonstrated that the inactivation process inactivates CAV.

Permanent records of mortality and of results of flock testing are kept for a minimum of five years. Details of any deterioration in egg preparation or hatchability, except for accidental cases identified as being of non-infectious origin, and of any test results indicating infection with a specified agent, are immediately submitted to the user of the eggs.

Chickens used for testing of the vaccine should be shown to be free from antibodies (Table 3).

2.7.8. Test for Absence of Non-Avian Mycoplasmas and Ureaplasmas

Culture media

Solid and liquid media suitable for sustaining the growth of a wide range of mycoplasmas are used. Each batch of media must be tested to show that it sustains the growth of *Mycoplasma hyopneumoniae* and *Ureaplasmas urealyticum*. For testing the media, the use of the low-passage strains is recommended.

No single medium may be satisfactory for optimal growth of the prescribed organisms. The solid and liquid media should be so selected that when used together they are capable of providing optimal growth condition for all likely contaminants. Liquid media should contain *phenol red*.

Method

Performed the test in the presence and in the absence of the preparation under examination. The preparation under examination should be shown to be free of any inhibitory effect on the test organisms. If necessary, any inhibitory effect should be neutralized and tests carried out to confirm neutralization of the inhibitory effect.

The number of containers recommended to be drawn for the test is 1 per cent of a batch, with a minimum of three and maximum of ten. The contents of the containers of a liquid vaccine under examination should be mixed. For a dried vaccine, the contents should be mixed after reconstitution.

Liquid media

Inoculate separately 50 ml of each medium with 5 ml of the test preparation under examination. If necessary after addition of the preparation under examination, the pH value of the liquid medium may be adjusted to the original value by the addition of sufficient *1M sodium hydroxide* or *1M hydrochloric acid*, as required. Incubate at 35° to 37° for 3 weeks observing three times a week.

Subculture by blind passage on the 3rd, 7th and 14th days after the beginning of incubation using an inoculum of 0.2 ml per agar plate of about 100 mm diameter. Observe the liquid media daily. If any colour change occurs, subculture immediately. If the culture in liquid medium shows bacterial or fungal contamination, repeat the test.

Using four plates for each subculture, incubate two of the plates at 35° to 37° under aerobic conditions in an atmosphere of air with high humidity and containing 5 to 10 per cent of carbon dioxide and the remaining two under anaerobic conditions in an atmosphere of *nitrogen* with high humidity and containing 5 to 10 per cent of *carbon dioxide*. Examine the plates regularly over a period of 3 weeks using a stereomicro-scope. Stain if necessary with a suitable stain.

Solid media

Inoculate each of four plates of medium with 0.2 ml of the test preparation under examination per agar plate of about 50 mm diameter. Incubate and examine the plates as described above.

If not more than one plate at each stage of the test is accidentally contaminatated with bacteria or fungi, or broken, and this happens not earlier than 7 days after inoculation, that plate may be ignored provided that on immediate examination it shows no evidence of mycoplasmal growth. If, at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or broken, the test has to be repeated.

Interpretation. The vaccine passes the test if there is no evidence of contamination with mycoplasmas or ureaplasmas in the test using liquid media as well as in the test using solid media.

2.7.9. Test for Absence of Avian Mycoplasmas in Live Viral Poultry Vaccines

Culture media

The solid and liquid media described here are used for the culture of most known mycoplasma species. The media selected must be tested at least to show that they sustain the growth of *Mycoplasma gallisepticum* (Medium A) and *Mycoplasma synoviae* (Medium B). For testing the media, the use of the low-passage strains is recommended.

No single medium may be satisfacotory for optimal growth of the prescribed organisms. Media other than the ones recommended below may be used provided that it has been demonstrated that each batch is capable of sustaining the growth of mycoplasmas in the presence and absence of the vaccine under examination.

A. Media recommended for the detection of *Mycoplasma gallisepticum*

Liquid medium

Beef heart infusion broth	900.0 ml
Horse serum (unheated)	20.0 ml
Yeast extract (25 per cent w/v)	10.0 ml
Thallium acetate (1 per cent w/v solution)	1.0 ml
Phenol red (0.06 per cent w/v solution)	5.0 ml
Benzylpenicillin potassium (or sodium) (1.2 per cent w/v solution)	0.25 ml
Deoxyribonucleic acid (0.2 per cent w/v solution)	1.2 ml

Mix and adjust to pH 7.8.

Solid medium

Prepare as described above but replacing beef heart infusion broth by 1.5 per cent w/v of agar in beef heat infusion broth.

Method

Perform the test in the presence and in the absence of the preparation under examination. The preparation under examination should be shown to be free of any inhibitory effect on the test organisms. If necessary, any inhibitory effect should be neutralized and tests carried out to confirm neutralization of the inhibitory effect.

Liquid Media

Use at least two liquid media suitable for the growth of mycoplasmas and use adequate quantities of the vaccine under examination for each medium. For a freeze-dried vaccine, reconstitute the contents of five containers or 5,000 doses of vaccine, whichever is less, in 12 ml of the liquid stated on the label or another suitable liquid. This is referred to as the 'test preparation' hereafter. For a liquid vaccine use an equivalent quantity. Inoculate 100 ml of each medium with 10 ml of the test preparation. If necessary, after addition of the test preparation, pH of the medium may be adjusted to the original value by the addition of sufficient *1M sodium hydroxide* or *1M hydrochloric acid*, as required. Incubate at 35° to 37° for 3 weeks observing three times a week. Subculture by blind passage on the 3rd, 7th and 14th days after the beginning of incubation using an inoculum of 0.2 ml per agar plate of about 100 mm diameter. If any colour change occurs, subculture immediately. If, the culture in liquid medium shows bacterial or fungal contamination, repeat the test.

Using four plates for each subculture, incubate two of the plates at 35° to 37° under aerobic conditions in an atmosphere of air with high humidity and containing 5 to 10 per cent of *carbon dioxide* and the remaining two under anaerobic conditions in an atmosphere of *nitrogen* with high humidity and containing 5 to 10 per cent of *carbon dioxide*. Examine the plates regularly over a period of 3 weeks using a stereomicroscope. Stain if necessary with a suitable stain and if growth occurs identify the isolate.

Solid media

Use at least two solid media suitable for the growth of mycoplasmas. Inoculate six plates of each medium with 0.2 ml of the test preparation described in the test with liquid media. Incubate and examine the plates as described above.

Interpretation. The vaccine passes the test if there is no evidence of contamination with mycoplasmas in the test using liquid media as well as in the test using solid media.

B. Media recommended for the detection of *Mycoplasma synoviae*

Liquid medium

Beef heart infusion broth	90.0 ml
Vitamin mixture	25.0 ml
Glucose (50 per cent w/v solution)	2.0 ml
Swine serum (inactivated at 56° for 30 minutes)	12.0 ml
β-Nicotinamide adenine dinucleotide (1 per cent w/v solution)	1.0 ml
Cysteine hydrochloride (1 per cent w/v solution)	1.0 ml
Phenol red (0.06 per cent w/w solution)	5.0 ml
Benzyl penicillin potassium (or sodium) (1.2 per cent w/v solution)	0.25 ml

Mix the solution of *β-nicotinamide adenine dinucleotide* and *cysteine hydrochloride*. After 10 minutes add the other ingredients. Mix and adjust to pH 7.8.

Solid medium

Beef heart infusion broth	90.0 ml
Ironagar	1.4 g
Vitamin mixture	0.025 ml
Glucose (50 per cent w/v solution)	2.0 ml
Swine serum (unheated)	12.0 ml
β-Nicotinamide adenine dinucleotide (1 per cent w/v solution)	1.0 ml
Cysteine hydrochloride (1 per cent w/v solution)	1.0 ml
Phenol red (0.06 per cent w/v solution)	5.0 ml
Benzyl penicillin potassium (or sodium) (1.2 per cent w/v solution)	0.25 ml

Mix beef heart infusion broth and ironagar, adjust to pH 7.8 and sterilize by autoclaving. Add the other ingredients to the mixture.

Special Reagents

Beef heart infusion broth

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

Vitamin mixture

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
Myo-Inositol	200 mg
Nicotinamde	100 mg
Pyridoxine hydrochloride	100 mg
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water	1000 ml

Ionagar

A highly refined agar for use in microbiology and immunology prepared by an ion-exchange procedure which results in a product having superior purity, clarity and gel strength.

It contains approximately:

Water	2.20 per cent
Ash	1.50 per cent
Acid-insoluble ash	0.20 per cent
Chlorine	None
Phosphate (calculated as P_2O_5)	0.30 per cent
Total nitrogen	0.30 per cent
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

2.7.10. Avian Viral Vaccines - Tests for Extraneous Agents in Seed Lots

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (2.7.10).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of cell cultures for the production of veterinary vaccines, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of two replicates are used, the total surface area and the total volume of test substance applied are not less than that

prescribed here and the survival rate requirements are adapted accordingly.

d) For a freeze-dried preparation, reconstitute using a suitable liquid. Unless otherwise stated or justified, the test substance must contain a quantity of virus equivalent to at least 10 doses of vaccine in 0.1 ml of inoculum.

e) If the virus of the seed lot would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

f) Monospecific antiserum and serum of avian origin used for cell culture or any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed here after under 13. Antibody specifications for sera used in extraneous agents testing.

g) Where specified in a monograph or otherwise justified, if neutralization of the virus of the seed lot is required but difficult to achieve, the *in vitro* tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent.

h) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.8.1) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. Test for extraneous agents using embryonated hen's eggs

Use a test substance, diluted if necessary containing a quantity of neutralised virus equivalent to at least 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test substance into 3 groups of 10 embryonated hens' eggs as follows:

Group 1	–	0.2 ml into the allantoic cavity of each 9 to 11 day-old embryonated egg;
Group 2	–	0.2 ml onto the chorio-allantoic membrane of each 9 to 11 day-old embryonated egg;
Group 3	–	0.2 ml into the yolk sac of each 5 to 6 day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 for 12 days. Discard embryos that die during the first 24 hours as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 hours after inoculation. Examine macroscopically for abnormalities in all embryos which die 24 hours after inoculation, or which survive the incubation period.

Examine also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos. Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The seed lot complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the seed lot and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of any extraneous agent.

2. Test in chicken kidney cells

Prepare seven monolayers of chicken kidney cells, each monolayer having an area of about 25 cm^2. Maintain two monolayers as negative controls and treat these in the same way as the five monolayers inoculated with the test substance, as described below.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test substance onto each of the five monolayers. Allow adsorption for 1 hour, add culture medium and incubate the cultures for a total of at least 21 days, subculturing at 4 to 7 day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle.

Inoculate 0.1 ml of pooled material onto each of five recently prepared monolayers of about 25 cm^2 each, at each passage. For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm^2 of cells from each of the monolayers for test A. The test is not valid if less than 80 per cent of the monolayer survives after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test substance. At the end of the total incubation period, carry out the following procedures.

Test A. Fix and stain (with *Giemsa* or *Haematoxylin* and *Eosin*) about 10 cm^2 of confluent cells from each of the five monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation or any other evidence of the presence of contaminating agents from the test substance.

Test B. Drain and wash about 25 cm^2 of cells from each of the fivemonolayers. Cover these cells with a 0.5 per cent suspension of washed chicken erythrocytes (using 1 ml of suspension for each 5 cm^2 of cells).

Incubate the cells at 4° for 20 minutes and then wash gently in *phosphate buffered saline pH 7.4*. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test substance.

Test C. Test pooled cell culture fluids using chicken erythrocytes for haemagglutination attributable to the presence of a haemagglutinating agent in the test substance.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

3. Test in chicken embryo fibroblast cells

Prepare seven monolayer of primary or secondary chicken embryo fibroblasts, from the tissues of 9 to 11 day-old embryos, each monolayer having an area of about 25 cm^2. Maintain two monolayer as negative controls and treat these in the same way as the five monolayer inoculated with the test vaccine, as described below.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayer. Allow adsorption for 1 hour and add culture medium. Incubate at 37° the cultures for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made with pooled cells and fluids from all five monolayer after carrying out a freeze-thaw cycle.

Inoculate 0.1 ml of pooled material onto each of five recently prepared monolayer of chicken embryofibroblast cells, each monolayer having an area of about 25 cm^2 each as before.

For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm^2 of cells from each of the monolayer, for test A. The test is not valid if less than 80 per cent of the test monolayer, or neither of the two negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test vaccine. At the end of the total incubation period, carry out the following procedures.

A. Fix and stain (with *Giemsa* or *Haematoxylin* and *Eosin*) about 10 cm^2 of confluent cells from each of the five original monolayer. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of a contaminating agent from the test vaccine.

B. Drain and wash about 25 cm^2 of cells from each of the five monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken red blood cells (using at least 1 ml of suspension for each 5 cm^2 of cells). Incubate the cells at 4° for 20 minutes and then wash gently in *phosphate buffered saline* pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test vaccine.

C. Test pooled cell culture fluids using chicken red blood cells for haemagglutination attributable to the presence of a haemagglutinating agent in the test vaccine.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

4. Test for avian leucosis viruses

Prepare at least 13 replicate monolayer of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11 day-old embryos that are known to be genetically susceptible to subgroups A, B and J of avian leucosis viruses and that support the growth of exogenous but not endogenous avian leucosis viruses (cells from C/E strain chickens are suitable). Each replicate shall have an area of about 50 cm^2.

Remove the culture medium when the cells reach confluence.

Inoculate 0.1 ml of the test substance onto each of five of the replicate monolayer. Allow adsorption for 1 hour, and add culture medium. Inoculate two of the replicate monolayers with subgroup A avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 ml), 2 with subgroup B avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 ml) and 2 with subgroup J avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 ml) as positive controls. Maintain not less than two non-inoculated replicate monolayer as negative controls.

Incubate the cells for a total period of 9 days, subculturing at 3 to 4 day intervals. Retain cells from each passage level and harvest the cells at the end of the total incubation period. Wash cells from each passage level from each replicate and resuspend the cells at 10^7 cells per ml in *barbital-buffered saline* for subsequent testing by a Complement Fixation for Avian Leucosis (COFAL) test or in *phosphate buffered saline* for testing by Enzyme-Linked Immunosorbent Assay (ELISA). Then, carry out three cycles of freezing and thawing to release any group-specific antigen and perform a COFAL test or an ELISA test on each extract to detect group-specific avian leucosis antigen if present.

The test is not valid if group-specific antigen is detected in less than five of the six positive control replicate monolayer or if a positive result is obtained in any of the negative control monolayer, or if the results for both of the two negative control monolayers are inconclusive. If the results for more than 1 of the test replicate monolayer are inconclusive, then further subcultures of reserved portions of the fibroblast monolayer

shall be made and tested until an unequivocal result is obtained. If a positive result is obtained for any of the test monolayer, then the presence of avian leucosis virus in the test substance has been detected.

The seed lot complies with the test if there is no evidence of the presence of any avian leucosis virus.

5. Test for avian reticuloendotheliosis virus

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11 day-old chick embryos or duck embryofibroblasts from the tissues of 9 to 11 day-old embryos, each monolayer having an area of about 25 cm^2.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of the test substance onto each of five of the monolayers. Allow adsorption for 1 hour and add culture medium. Inoculate four of the monolayers with avian eticuloendotheliosis virus as positive controls (not more than 10 $CCID_{50}$ in 0.1 ml). Maintain two non-inoculated monolayers as negative controls.

Incubate the cells for a total of 10 days, subculturing twice at 3 to 4 day intervals. The test is not valid if less than three of the four positive controls or less than four of the five test monolayers or neither of the two negative controls survive after any passage.

For the last subculture, grow the fibroblasts on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent fibroblasts from each of the original 11 monolayers for the subsequent test: test about 10 cm^2 of confluent fibroblasts derived from each of the original 11 monolayers by immunostaining for the presence of avian reticuloendotheliosis virus. The test is not valid if avian reticuloendotheliosis virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers, or if the results for both of the two negative control monolayers are inconclusive. If the results for more than one of the test mono layers are inconclusive then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of avian reticuloendotheliosis virus.

6. Test for chicken infection anaemia (CIA) virus

Prepare eleven 20 ml suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 ml cell culture flasks containing about 5 x 10^5 cells per ml. Inoculate 0.1 ml of test substance into each of five flasks. Inoculate four of the suspensions with 10 $CCID_{50}$ CIA as positive controls. Maintain not less than two non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3 to 4 day intervals.

During the subculturing the presence of CIA may be indicated by a metabolic colour change in the infected cultures, the culture fluids become red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed and resuspend at about 5 x 10^5 cells per ml and place 25 ml in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if CIA virus is detected in less than three of the four positive controls or in any of the non-inoculated controls. If the results for more than one of the test suspensions are inconclusive, then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of CIA virus.

7. Test for egg drop syndrome virus

Prepare 11 monolayer of chicken embryo liver cells, from the tissues of 14 to 16 day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of five of the monolayer (test monolayer).

Allow adsorption for 1 hour at 37°, add culture medium. Inoculate four of the monolayer with a suitable strain of egg drop syndrome virus (not more than 10 $CCID_{50}$ in 0.1 ml to serve as positive control monolayer. Maintain two non-inoculated monolayer as negative control monolayer.

Incubate the cells for a total of at least 21 days, subculturing every 4 to 5 days. Each passage is made as follows: Carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayer, from the positive control monolayer and from the negative control monolayer; Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayer of chicken embryo liver cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the two negative control monolayer survive after any passage.

Examine microscopically all the cell cultures at frequent intervals throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedure.

Test separately, cell culture fluid from the test monolayer, positive control monolayer and negative control monolayer, using chicken red blood cells, for haemagglutination attributable to the presence of haemagglutinating agents.

The test is not valid if egg drop syndrome virus is detected in less than three of the four positive control monolayer or in

any of the negative control monolayer or if the results for both of the two negative control monolayer are inconclusive.

If the results for more than one of the test monolayer are inconclusive then further subcultures of reserved portions of the monolayer shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of egg drop syndrome virus or any other extraneous agent.

8. Test for Marek's Disease Virus

Prepare 11 monolayer of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11 day old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence.

Inoculate 0.1 ml of test vaccine onto each of five of the monolayer (test mono layer). Allow adsorption for 1 hour, and add culture medium. Inoculate four of the monolayer with a suitable strain of Marek's disease virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayer as negative controls.

Incubate the cultures for a total of 21 days, subculturing at four to five day intervals. Each passage is made as follows:

Trypsinise the cells, prepare separate pools of the cells from the test monolayer, from the positive control monolayer and from the negative control monolayer. Mix an appropriate quantity of each with a suspension of freshly prepared primary or secondary chick embryo fibroblasts and prepare 5, 4 and 2 monolayer, as before. The test is not valid if less than four of the five test monolayer or less than three of the four positive controls or neither of the two negative control monolayer survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 25 cm^2 of confluent cells from each of the original 11 monolayer for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayer by immunostaining for the presence of Marek's disease virus. The test is not valid if Marek's disease virus is detected in less than three of the four positive control monolayer or in any of the negative control monolayer, or if the results for both of the two negative control monolayer are inconclusive.

The seed lot complies with the test if there is no evidence of the presence of Marek's disease virus or any other extraneous agent.

9. Tests for Turkey rhinotracheitis virus

A. In chicken embryo fibroblasts

NOTE—This test can be combined with Test 2 by using the same test monolayers and negative controls, for all stages up to the final specific test for Turkey rhinotracheitis virus on cells prepared from the last subculture.

Prepare 11 monolayer of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11 day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of five of the monolayer (test monolayer). Allow adsorption for 1 hour at 37°, and add culture medium. Inoculate four of the monolayer with a suitable strain of Turkey rhinotracheitis virus as positive controls (not more than 10 $CCID_{50}$ in 0.1 ml). Maintain two non-inoculated monolayer as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayer, from the positive control monolayer and from the negative control monolayer, inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayer of chicken embryo fibroblasts cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than four of the five test monolayer or less than three of the four positive controls or neither of the two negative control monolayer survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original elven monolayer for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayer by immunostaining for the presence of Turkey rhinotracheitis virus. The test is not valid if Turkey rhinotracheitis virus is detected in less than three of the four positive control monolayer or in any of the negative control monolayer or if the results for both of the two negative control monolayer are inconclusive. If the results for both of the two test monolayer are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of Turkey rhinotracheitis virus or any other extraneous agent.

B. In vero cells

Prepare even monolayer of Vero cells, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayer (test monolayer). Allow adsorption for 1 hour at 37° and add culture medium. Inoculate 4 of the

monolayers with a suitable strain of Turkey rhinotracheitis virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayer as negative controls.

Incubate at 37° the cultures for a total of at least 21 days, subculturing at four to five day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayer, from the positive control monolayer and from the negative control monolayer. Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayer of Vero cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than 4 of the 5 test monolayer or less than thre of the four positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayer for the subsequent test. Test about 10 cm^2 of confluent cells derived from each of the original elven monolayer by immunostaining for the presence of Turkey rhinotracheitis virus. The test is not valid if Turkey rhinotracheitis virus is detected in less than three of the four positive control monolayer or in any of the negative control monolayer, or if the results for both of the two negative control monolayer are inconclusive. If the results for more than oneof the test monolayer are inconclusive then further subcultures of reserved portions of the monolayer shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of Turkey rhinotracheitis virus or any other extraneous agent.

10. Test for duck enteritis virus

This test is carried out for vaccines prepared on duck or goose substrates. Prepare 11 monolayer of primary or secondary Muscovy duck embryo liver cells, from the tissues of 21 or 2 2 day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence.

Inoculate 0.1 ml of test vaccine onto each of five of the monolayer (test monolayer). Allow adsorption for 1 hour at 37° and add culture medium. Inoculate four of the monolayer with a suitable strain of duck enteritis virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayer as negative controls.

Incubate the cultures at 37° for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Trypsinise the cells and prepare separate pools of the cells from the test monolayer, from the positive control monolayer and from the negative control monolayer. Mix a portion of each with a suspension of freshly prepared primary or secondary Muscovy duck embryo liver cells to prepare 5, 4 and two monolayer, as before. The test is not valid if less than four of the five test monolayer or less than 3 of the 4 positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayer for the subsequent test. Test about 10 cm^2 of confluent cells derived from each of the original elven monolayer by immunostaining for the presence of duck enteritis virus. The test is not valid if duck enteritis virus is detected in less than three of the four positive control monolayer or in any of the negative control monolayer, or if the results for both of the two negative control monolayer are inconclusive. If the results for more than one of the test monolayer are inconclusive then further subcultures of reserved portions of the monolayer shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of duck enteritis virus or any other extraneous agent.

11. Test for duck and goose parvoviruses

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare a suspension of sufficient primary or secondary Muscovy duck embryo fibroblasts from the tissues of 16 to 18 day-old embryos, to obtain not less than 11 monolayer, each having an area of about 25 cm^2. Inoculate 0.5 ml of test vaccine into an aliquot of cells for five monolayer and seed into five replicate containers to form five test monolayer.

Inoculate 0.4 ml of a suitable strain of duck parvovirus (not more than 10 $CCID_{50}$ in 0.1 ml) into an aliquot of cells for four monolayer and seed into 4 replicate containers to form four positive control monolayer. Prepare two non-inoculated monolayer as negative controls.

Incubate the cultures at 37° for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayer, from the positive control monolayer and from the negative control monolayer. Inoculate 0.5 ml, 0.4 ml and 0.2 ml of the pooled materials into aliquots of a fresh suspension of sufficient primary or secondary Muscovy duck embryo fibroblast cells to prepare 5, 4 and 2 monolayer, as before.

The test is not valid if less than four of the five test monolayer or less than three of the four positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayer for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayer by immunostaining for the presence of duck or goose parvovirus. The test is not valid if duck parvovirus is detected in less than three of the four positive control monolayer or in any of the negative control monolayer, or if the results for both of the two negative control monolayer are inconclusive.

The seed lot complies with the test if there is no evidence of the presence of duck (or goose) parvovirus or any other extraneous agent.

12. Test for extraneous agents using chicks

Inoculate each of at least 10 chicks, with the equivalent of 100 doses of vaccine by the intramuscular route and with the equivalent of 10 doses by eye-drop. Chicks that are two weeks of age are used in the test except that if the seed virus is pathogenic for birds of this age, older birds may be used, if required and justified. In exceptional cases, for inactivated vaccines, the virus may be neutralized by specific antiserum if the seed virus is pathogenic for birds at the age of administration. Repeat these inoculations two weeks later. Observe the chicks for a period of five weeks from the day of the first inoculation. No antimicrobial agents shall be administered to the chicks during the test period. The test is not valid if less than 80 per cent of the chicks survive to the end of the test period.

Collect serum from each chick at the end of the test period. Test each serum sample for antibodies against each of the agents listed below (with the exception of the virus type of the seed lot) using one of the methods indicated for testing for the agent.

A. Standard test

Agent	Type of Test
Avian adenoviruses, group	1 SN, EIA, AGP
Avian encephalomyelitis virus	AGP, EIA
Avian infectious bronchitis virus	EIA, HI
Avian infectious laryngotracheitis virus	SN, EIA, IS
Avian leucosis viruses	SN,EIA,IS
Avian nephritis virus	IS
Avian reoviruses	IS, EIA
Avian reticuloendotheliosis virus	AGP, IS, EIA
Chicken anaemia virus	IS, EIA, SN
Egg drop syndrome virus	HI, EIA
Avian Infectious bursal disease virus	GP, EIA
Influenza A virus	AGP, EIA
Marek's disease virus	AGP
Newcastle disease virus	HI, EIA
Turkey rhinotracheitis virus	EIA
Mycoplasma gallisepticum	Agg and to confirm positive test HI
Mycoplasma synovial	Agg and to confirm positive test HI
Salmonella pullorum	Agg

Agg : agglutination
AGP : agar gel precipitation
EIA : enzyme immunoassay (e.g. ELISA)
IS : immunostaining (e.g. fluorescent antibody)
HI : haemagglutination inhibition
SN : serum neutralisation

B. Additional tests for Turkey extraneous agents

If the seed virus is of Turkey origin or was propagated in Turkey substrates, tests for antibodies against the following agents are also carried out.

Agent	Type of test
Chlamydia spp.	EIA
Avian infectious haemorrhagic enteritis virus	AGP
Avian paramyxovirus 3	HI
Avian infectious bursal disease virus type 2	SN

A test for freedom from Turkey lympho-proliferative disease virus is carried out by intraperitoneal inoculation of twenty four week-old Turkey poults. Observe the poults for 40 days. The test is not valid if more than 20 per cent of the poults die from non-specific causes. The seed lot complies with the test if sections of spleen and thymus taken from 10 poults two weeks after inoculation show no macroscopic or microscopic lesions (other than those attributable to the seed lot virus) and no poult dies from causes attributable to the seed lot.

C. Additional tests for duck extraneous agents

If the seed virus is of duck origin or was propagated in duck substrates. Tests for antibodies against the following agents are also carried out.

Agent	Type of test
Chlamydia spp.	EIA
Duck and Goose parvoviruses	SN
Duck enteritis virus	SN
Duck hepatitis virus type I	SN

The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

The test is not valid if antibodies are detected in the chicks to any of the test agents before inoculation.

Clinical signs of disease in the chicks during the test period (other than signs attributable to the virus of the seed lot) and the detection of antibodies in the chicks after inoculation, (with the exception of antibodies to the virus of the seed lot) are classed as evidence of the presence of an extraneous agent in the seed lot.

It is recommended that sera from these birds is retained so that additional testing may be carried out if requirements change.

13. Antibody specifications for sera used in extraneous agents testing

All batches of serum to be used in extraneous agents testing either to neutralise the vaccine virus (seed lot or batch of finished product) or all batches of avian serum used as a supplement for culture media used for tissue culture propagation, shall be shown to be free of antibodies against and free from inhibitory effects on the following micro-organisms by suitably sensitive tests:

Avian adenoviruses

Avian encephalomyelitis virus

Avian infectious bronchitis viruses

Avian infectious bursal disease virus types 1 and 2

Avian infectious haemorrhagic enteritis virus

Avian infectious laryngotracheitis virus

Avian leucosis viruses

Avian nephritis virus

Avian paramyxoviruses 1 to 9

Avian reoviruses

Avian reticuloendotheliosis virus

Chicken anaemia virus

Duck enteritis virus

Duck hepatitis virus type I

Egg drop syndrome virus

Fowl pox virus

Influenza viruses

Marek's disease virus

Turkey herpesvirus

Turkey rhinotracheitis virus

Non-immune serum for addition to culture media can be assumed to be free of antibodies against any of these viruses if the agent is known not to infect the species of origin of the serum and it is not necessary to test the serum for such antibodies. Monospecific antisera for virus neutralisation can be assumed to be free of the antibodies against any of these viruses if it can be shown that the immunising antigen could not have been contaminated with antigens derived from that virus and if the virus is known not to infect the species of origin of the serum; it is not necessary to test the serum for such antibodies. It is not necessary to retest sera obtained from birds from SPF chicken flocks (2.7.7).

Batches of sera prepared for neutralising the vaccine virus must be prepared from any passage level derived from the virus isolate used to prepare the master seed lot or from an isolate cultured in the same cell line.

2.7.11. Avian Live Virus Vaccines - Tests for Extraneous Agents in Batches of Finished Products

In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (2.7.7).

a) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of cell cultures for the production of veterinary vaccines with the exception of the karyotype test and the tumorigenicity test which do not have to be carried out.

b) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of vaccine test applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

c) In these tests, use the liquid vaccine or reconstitute a quantity of the freeze-dried preparation under test with the liquid stated on the label or another suitable diluent such as *water for injections*. Unless otherwise stated or justified, the test substance contains the equivalent of 10 doses in 0.1 ml of inoculum.

d) If the vaccine virus would interfere with the conduct and sensitivity of the test, neutralize the virus in the preparation with a monospecific antiserum.

e) Where specified in a monograph or otherwise justified, if neutralization of the vaccine virus is required but difficult to achieve, the *in vitro* tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to *in vitro* tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch

of vaccine, as described under 6. Test for extraneous agents using chicks of chapter (2.7.10).

f) Monospecific antiserum and serum of avian origin used for cell culture and any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed under 7. Antibody specifications for sera used in extraneous agents testing (2.7.10).

g) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.8.1) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. Test for extraneous agents using embryonated hen's eggs

Prepare the test vaccine, diluted if necessary, to contain neutralised virus equivalent to 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test vaccine into 3 groups of 10 embryonated hen's eggs as follows:

Group 1	–	0.2 ml into the allantoic cavity of each 9 to 11 day-old embryonated egg;
Group 2	–	0.2 ml onto the chorio-allantoic membrane of each 9 to 11 day-old embryonated egg;
Group 3	–	0.2 ml into the yolk sac of each 5 to 6 day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 for 12 days. Discard embryos that die during the first 24 hours as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 hours after inoculation. Examine macroscopically for abnormalities all embryos which die more than 24 hours after inoculation, or which survive the incubation period.

Examine also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos.

Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The batch of vaccine complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the vaccine and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of extraneous agents.

2. Test in chicken embryo fibroblast cells

Prepare 7 monolayers of primary or secondary chicken embryo fibroblasts, from the tissues of 9 to 11day-old embryos, each monolayer having an area of about 25 cm^2. Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test vaccine, as described below.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers. Allow adsorption for 1 hour and add culture medium. Incubate at 37° the cultures for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle.

Inoculate 0.1 ml of pooled material onto each of 5 recently prepared monolayers of chicken embryofibroblast cells, each monolayer having an area of about 25 cm^2 each as before.

For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm^2 of cells from each of the monolayers, for test A. The test is not valid if less than 80 per cent of the test monolayers, or neither of the two negative control mono layers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test vaccine. At the end of the total incubation period, carry out the following procedures.

A. Fix and stain (with *Giemsa* or *Haematoxylin* and *Eosin*) about 10 cm^2 of confluent cells from each of the five original monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of a contaminating agent from the test vaccine.

B. Drain and wash about 25 cm^2 of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken red blood cells (using at least 1 ml of suspension for each 5 cm^2 of cells). Incubate the cells at 4° for 20 minutes and then wash gently in *phosphate buffered saline pH 7.4*. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test vaccine.

C. Test pooled cell culture fluids using chicken red blood cells for haemagglutination attributable to the presence of a haemagglutinating agent in the test vaccine.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The batch of vaccine complies

with the test if there is no evidence of the presence of any extraneous agent.

3. Test for egg drop syndrome virus

Prepare 11 monolayers of chicken embryo liver cells, from the tissues of 14 to 16 day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers).

Allow adsorption for 1 hours at 37°, add culture medium. Inoculate 4 of the monolayers with a suitable strain of egg drop syndrome virus (not more than 10 $CCID_{50}$ in 0.1 ml to serve as positive control monolayers. Maintain two non-inoculated monolayers as negative control monolayers.

Incubate the cells for a total of at least 21 days, subculturing every 4 to 5 days. Each passage is made as follows: Carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo liver cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures at frequent intervals throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedure.

Test separately, cell culture fluid from the test monolayers, positive control monolayers and negative control monolayers, using chicken red blood cells, for haemagglutination attributable to the presence of haemagglutinating agents.

The test is not valid if egg drop syndrome virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers or if the results for both of the two negative control monolayers are inconclusive.

If the results for more than one of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of egg drop syndrome virus or any other extraneous agent.

4. Test for Marek's disease virus

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11 day old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence.

Inoculate 0.1 ml of test vaccine onto each of five of the monolayers (test mono layers). Allow adsorption for 1 hour, and add culture medium. Inoculate four of the monolayers with a suitable strain of Marek's disease virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayers as negative controls.

Incubate the cultures for a total of 21 days, subculturing at 4 to 5 day intervals. Each passage is mild as follows:

Trypsinise the cells, prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix an appropriate quantity of each with a suspension of freshly prepared primary or secondary chick embryo fibroblasts and prepare 5, 4 and 2 monolayers, as before. The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the two negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 25 cm^2 of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Marek's disease virus. The test is not valid if Marek's disease virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers, or if the results for both of the two negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of Marek's disease virus or any other extraneous agent.

5. Tests for Turkey rhinotracheitis virus

A. In chicken embryo fibroblasts

NOTE –This test can be combined with Test 2 by using the same test monolayers and negative controls, for all stages up to the final specific test for Turkey rhinotracheitis virus on cells prepared from the last subculture.

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9 to 11day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of five of the monolayers (test monolayers). Allow adsorption for 1 hour at 37°, and add

culture medium. Inoculate four of the monolayers with a suitable strain of Turkey rhinotracheitis virus as positive controls (not more than 10 $CCID_{50}$ in 0.1 ml). Maintain two non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo fibroblasts cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the two negative control monolayers survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Turkey rhinotracheitis virus. The test is not valid if Turkey rhinotracheitis virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers or if the results for both of the two negative control monolayers are inconclusive. If the results for both of the two test monolayers are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of Turkey rhinotracheitis virus or any other extraneous agent.

B. In vero cells

Prepare 11 monolayers of Vero cells, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of five of the monolayers (test monolayers). Allow adsorption for 1 hour at 37°, and add culture medium. Inoculate four of the monolayers with a suitable strain of Turkey rhinotracheitis virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayers as negative controls.

Incubate at 37° the cultures for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of Vero cells, each monolayer having an area of about 25 cm^2 as before. The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayers for the subsequent test. Test about 10 cm^2 of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Turkey rhinotracheitis virus. The test is not valid if Turkey rhinotracheitis virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers, or if the results for both of the two negative control monolayers are inconclusive. If the results for more than one of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of Turkey rhinotracheitis virus or any other extraneous agent.

6. Test for chicken anaemia virus

Prepare eleven 20 ml suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 ml flasks containing about 5 x 10^5 cells per ml. Inoculate 0.1 ml of test vaccine into each of five of these flasks. Inoculate four other suspensions with 10 $CCID_{50}$ chicken anaemia virus as positive controls. Maintain not less than two non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing eight times at 3 to 4 day intervals.

During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids becoming red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed, resuspend at about 10^6 cells per ml and place 25 µl in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in less than three of the four positive controls or in any of the non-inoculated controls. If the results for more than one of the test suspensions are inconclusive then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained. The batch of vaccine complies with the test if there is no evidence of the presence of chicken anaemia virus.

7. Test for duck enteritis virus

This test is carried out for vaccines prepared on duck or goose substrates. Prepare 11 monolayers of primary or secondary

Muscovy duck embryo liver cells, from the tissues of 21 or 22 day-old embryos, each monolayer having an area of about 25 cm^2. Remove the culture medium when the cells reach confluence.

Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 hour at 37° and add culture medium. Inoculate four of the monolayers with a suitable strain of duck enteritis virus (not more than 10 $CCID_{50}$ in 0.1 ml) to serve as positive controls. Maintain two non-inoculated monolayers as negative controls.

Incubate the cultures at 37° for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Trypsinise the cells and prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix a portion of each with a suspension of freshly prepared primary or secondary Muscovy duck embryo liver cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if less than 4 of the 5 test monolayers or less than three of the four positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayers for the subsequent test. Test about 10 cm^2 of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck enteritis virus. The test is not valid if duck enteritis virus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers, or if the results for both of the two negative control monolayers are inconclusive. If the results for more than one of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of duck enteritis virus or any other extraneous agent.

8. Test for duck and goose parvoviruses

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare a suspension of sufficient primary or secondary Muscovy duck embryo fibroblasts from the tissues of 16 to 18 day-old embryos, to obtain not less than 11 monolayers, each having an area of about 25 cm^2. Inoculate 0.5 ml of test vaccine into an aliquot of cells for five monolayers and seed into five replicate containers to form five test monolayers.

Inoculate 0.4 ml of a suitable strain of duck parvovirus (not more than 10 $CCID_{50}$ in 0.1 ml) into an aliquot of cells for four monolayers and seed into four replicate containers to form four positive control monolayers. Prepare two non-inoculated monolayers as negative controls.

Incubate the cultures at 37° for a total of at least 21 days, subculturing at 4 to 5 day intervals. Each passage is made as follows:

Carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.5 ml 0.4 ml and 0.2 ml of the pooled materials into aliquots of a fresh suspension of sufficient primary or secondary Muscovy duck embryo fibroblast cells to prepare 5, 4 and 2 monolayers, as before.

The test is not valid if less than four of the five test monolayers or less than three of the four positive controls or neither of the two negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm^2 of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm^2 of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck or goose parvovirus. The test is not valid if duck parvovirus is detected in less than three of the four positive control monolayers or in any of the negative control monolayers, or if the results for both of the two negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of duck (or goose) parvovirus or any other extraneous agent.

2.7.12. Evaluation of Efficacy of Vaccines and Immunosera

During development of a vaccine or immunosera, tests are carried out to demonstrate that the product is efficacious when administered by each of the recommended routes and methods of administration and using the recommended schedule to animals of each species and category for which use of the product is to be recommended. The type of efficacy testing to be carried out varies depending on the particular type of product.

During development of the product, the tests described in the Production section of a monograph may be carried out; the following must be taken into account:

The dose to be used is that quantity of the product to be recommended for use and containing the minimum titre or potency expected at the end of the period of validity.

For live vaccines, use vaccine containing virus/bacteria at the most attenuated passage level that will be present in a batch of vaccine.

For immunosera, if appropriate, the dose tested also contains minimum quantities of immunoglobulin or gammaglobulin and/or total protein.

The efficacy evidence must support all the claims being made. Where it is claimed that there is protection from infection this must be demonstrated using re-isolation techniques. If more than one claim is made, supporting evidence for each claim is required.

Vaccines. The influence of passively acquired and maternally derived antibodies on the efficacy of a vaccine is adequately evaluated. Any claims, stated or implied, regarding onset and duration of protection shall be supported by data from trials.

The efficacy of each of the components of multivalent and combined vaccines shall be demonstrated using the combined vaccine.

Immunosera. Particular attention must be paid to providing supporting data for the efficacy of the regime that is to be recommended. Any claim, stated or implied, regarding onset and duration of protection or therapeutic effect must be supported by data from trials. For example, the duration of the protection afforded by a prophylactic dose of an antiserum must be studied so that appropriate guidance for the user can be given on the label.

Studies of immunological compatibility are undertaken when simultaneous administration is recommended or where it is a part of a usual administration schedule. Wherever a product is recommended as part of an administration scheme, the priming or booster effect or the contribution of the product to the efficacy of the scheme as a whole is demonstrated.

Tests

In principle, demonstration of efficacy is undertaken under well-controlled laboratory conditions by challenge of the target animal under the recommended conditions of use.

To the extent possible, the conditions under which the challenge is carried out shall be close to the natural conditions for infection, for example with regard to the amount of challenge organism and the route of administration of the challenge.

Vaccines. Unless otherwise justified, challenge is carried out using a strain different from the one used in the production of the vaccine.

If possible, the immune mechanism (cell-mediated/humoral, local/general, classes of immunoglobulin) that is initiated after the administration of the vaccine to target animals shall be determined.

Immunosera. Data are provided from measurements of the antibody levels achieved in the target species after administration of the product, as recommended. Where suitable published data exist, references are provided to relevant published literature on protective antibody levels and challenge studies are avoided.

Where challenges are required, these can be given before or after administration of the product, in accordance with the indications and specific claims to be made.

Field trials. In general, results from laboratory tests are supplemented with data from field trials, carried out, unless otherwise justified, with untreated control animals. Provided that laboratory tests have adequately assessed the safety and efficacy of a product under experimental conditions using vaccines of maximum and minimum titre or potency respectively, a single batch of product could be used to assess both safety and efficacy under field conditions. In these cases, a typical routine batch of intermediate titre or potency may be used. Where laboratory trials cannot be supportive of efficacy, the performance of field trials alone may be acceptable.

2.7.13. Cell Cultures for the Production of Veterinary Vaccines

Cell cultures for the production of vaccines for veterinary use comply with the requirements of this section. It may also be necessary that cell cultures used for testing of vaccines for veterinary use also comply with some or all of these requirements. For most mammalian viruses, propagation in cell lines is possible and the use of primary cells is then not acceptable. Permanently infected cells used for production of veterinary vaccines comply with the appropriate requirements described below. The cells shall be shown to be infected only with the agent stated.

Cell lines

Cell lines are normally handled according to a cell-seed system. Each master cell seed is assigned a specific code for identification purposes. The master cell seed is stored in aliquots at -70° or lower. Production of vaccine is not normally undertaken on cells more than twenty passages from the master cell seed. Where suspension cultures are used, an increase in cell numbers equivalent to approximately three population doublings is considered equivalent to one passage. If cells beyond twenty passage levels are to be used for production, it shall be demonstrated, by validation or further testing, that the production cell cultures are essentially similar to the master cell seed with regard to their biological characteristics and purity and that the use of such cells has no deleterious effect on vaccine production.

The history of the cell line shall be known and recorded in detail (for example, origin, number of passages and media used for multiplication, storage conditions). The method of storing and using the cells, including details of how it is ensured that

the maximum number of passages permitted is not exceeded during product manufacture, are recorded. A sufficient quantity of the master cell seed and each working cell seed are kept for analytical purposes.

The tests described below are carried out (as prescribed in Table.1) on a culture of the master cell seed and the working cell seed or on cell cultures from the working cell seed at the highest passage level used for production and derived from a homogeneous sample demonstrated to be representative.

Characteristics of Culture

The appearance of cell monolayer, before and after histological staining, is described. Information, if possible numerical data, is provided especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

Karyotype

A chromosomal examination is made of not less than fifty cells undergoing mitosis in the master cell seed and at a passage level at least as high as that to be used in production. Any chromosomal marker present in the master cell seed must also be found in the high passage cells and the modal number of chromosomes in these cells must not be more than 15 per cent higher than of cells of the master cell seed. The karyotypes must be identical. If the modal number exceeds the level stated, if the chromosomal markers are not found in the working cell seed at the highest level used for production or if the karyotype differs, the cell line shall not be used for manufacture.

Table 1 – *Cell culture stage at which test are carried out*

	Mater cell seed	Working cell seed	Cell from working cell seed at highest passage level
General microscopy	+	+	+
Sterility	+	+	-
Mycoplasmas	+	+	-
Viruses	+	+	-
Identification of species	+	-	+
Karyotype	+	-	+
Tumorigenicity	+	-	-

Identification of the Species

It shall be shown, by one validated method that the master cell seed and the cells from the working cell seed at the highest passage level used for production come from the species of origin specified. When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

Sterility (2.2.11). The cells comply with the test for sterility. The sample of cells under examined consists of not less than the number of cells in a monolayer with an area of 70 cm^2 or, for cells grown in suspension, an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Mycoplasmas (2.7.4). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Absence of Contaminating Viruses. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below, are carried out. The monolayer tested shall have an area of at least 70 cm^2, and shall be prepared and maintained using medium and additives, and grown under similar conditions to those used for the preparation of the vaccine. The monolayers are maintained in culture for a total of at least 28 days.

Subcultures are made at 7 days intervals, unless the cells do not survive for this length of time, when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers, are produced for the final subculture to carry out the tests specified below.

The monolayer are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by *immunofluorescence* and other suitable tests as indicated below.

Detection of Cytopathic Viruses. Two monolayer of at least 6 cm^2 each are stained with an appropriate cytological stain. The entire area of each stained monolayer is examined for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality which might be attributable to a contaminant.

Detection of Haemadsorbent Viruses. Monolayers totalling at least 70 cm^2 are washed several times with an appropriate buffer and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times cells are examined for the presence of haemadsorption.

Detection of Specified Viruses. Tests are carried out for freedom from contaminants specific for the species of origin of the cell line and for the species for which the product is intended. Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to

suitable tests, for example using fluorescein- conjugated antibodies or similar reagents.

Tests in Other Cell Cultures

Monolayers totalling at least 140 cm^2 are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Inoculate aliquots onto the following cells at any time up to 70 per cent confluency: primary cells of the source species; cells sensitive to viruses pathogenic for the species for which the vaccine is intended; cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze- thawed extracts are prepared as above and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. The cultures are examined regularly for the presence of any cytopathic changes indicative of living organisms.

At the end of this period of 14 days, the inoculated cells are subjected to the following checks: freedom from cytopathic and haemadsorbent organisms, using the methods specified in the relevant paragraphs above; absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on Detection of Specified Viruses.

Tumorigenicity. The risk of a cell line for the target species must be evaluated and, if necessary, a test is carried out.

Primary Cells. For most mammalian vaccines, the use of primary cells is not acceptable for the manufacture of vaccines since cell lines can be used. If there is no alternative to the use of primary cells, the cells are obtained from a herd or flock free from specified pathogens, with complete protection from introduction of diseases (for example, disease barriers, filters on air inlets, suitable quarantine before introduction of animals). Chicken flocks comply with the requirements prescribed in general chapter (2.7.7) *Chicken Flocks Free from Specified Pathogens for the Production and Quality Control of Vaccines*. For all other species, the herd or flock is shown to be free from relevant specified pathogens. All the breeding stock in the herd or flock intended to be used to produce primary cells for vaccine manufacture is subject to a suitable monitoring procedure including regular serological checks carried out at least twice a year and two supplementary serological examinations performed in 15 per cent of the breeding stock in the herd between the two checks mentioned above.

Wherever possible, particularly for mammalian cells, a seed-lot system is used with, for example, a master cell seed formed after less than five passages, the working cell seed being no more than five passages from the initial preparation of the cell suspension from the animal tissues.

Each master cell seed, working cell seed and cells of the highest passage of primary cells are checked in accordance with Table 2 and the procedure described below. The sample tested shall cover all the sources of cells used for the manufacture of the batch. No batches of vaccine manufactured using the cells may be released if any one of the checks performed produces unsatisfactory results.

Table 2 – *Cell culture stage at which test are carried out*

	Mater cell seed	Working cell seed	Cell from working cell seed at highest passage level
General microscopy	+	+	+
Sterility	+	+	-
Mycoplasma	+	+	-
Viruses	+	+	-
Identification of species	+	-	-

Characteristics of Cultures .

The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is recorded, especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

Identification of Species. It shall be demonstrated by one validated test that the master cell seed comes from the specified species of origin. When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

Sterility (2.2.11). The cells comply with the test for sterility. The sample of cells under examined consists of not less than the number of cells in a monolayer with an area of 70 cm^2 or for cells grown in suspension an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Mycoplasmas (2.7.4). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Absence of Contaminating Viruses. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below are carried out. The monolayer tested shall be at least 70 cm^2, and shall be prepared and maintained in culture using the same medium and additives, and under similar conditions to those used for the preparation of the vaccine.

The monolayers are maintained in culture for a total of at least 28 days or for the longest period possible if culture for 28 days is impossible. Subcultures are made at 7day intervals, unless the cells do not survive for this length of time when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers are produced for the final subculture to carry out the tests specified below. The monolayer are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immunofluorescence and other suitable tests as indicated below.

Detection of Cytopathic Viruses. Two monolayer of at least 6 cm^2 each are stained with an appropriate cytological stain. Examine the entire area of each stained monolayer for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality that might be attributable to a contaminant.

Detection of Haemadsorbent Viruses. Monolayers totalling at least 70 cm^2 are washed several times with a suitable buffer solution and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times, examine cells for the presence of haemadsorption.

Detection of Specified Viruses. Tests are being carried out for freedom of contaminants specific for the species of origin of the cells and for the species for which the product is intended. Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to suitable tests using fluorescein-conjugated antibodies or similar reagents.

Tests in Other Cell Cultures

Monolayers totalling at least 140 cm^2 are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Aliquots are inoculated onto the following cells at any time up to 70 per cent confluency; primary cells of the source species; cells sensitive to viruses pathogenic for the species for which the vaccine is intended; cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze- thawed extracts are prepared as above, and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. All cultures are regularly examined for the presence of any cytopathic changes indicative of living organisms. At the end of this period of 14 days, the inoculated cells are subjected to the following checks. Freedom from cytopathic and haemadsorbent organisms is demonstrated using the methods specified in the relevant paragraphs above; relevant substrates are tested for the absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on detection of specified viruses.

2.7.14. Test for absence of Mycobacteria

Method A

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurylsulfate solution. Inoculate 0.2 ml of the sample in triplicate onto each of 2 suitable solid media *(Lowenstein-Jensen medium* and *Middle brook 7H10 medium* are considered suitable). Inoculate 0.5 ml in triplicate into a suitable liquid medium. Incubate all media at 37° for 56 days. Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a Mycobacterium sp. such as BCG and if necessary use a suitable neutralising substance. If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test. If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

Method B

Nucleic Acid Amplification Techniques (NAT) (2.8.1) may be used as an alternative to this culture method provided such an assay is validated and shown to be comparable to the culture method.

2.7.15. Estimation of Total and Free PRP content of Haemophilus Influenzae type b vaccine using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The total PRP concentration is not less than 80.0 percent and not more than 120.0 percent of the stated amount on the label. The amount of Free PRP is not more than that approved for the particular product

Total PRP and free PRP content. Determine by liquid Chromatography (2.4.14)

Buffer solution. A solution prepared by mixing 12.8 ml of *0.2 M sodium dihydrogen phosphate monohydrate* and 12.3 ml of *0.2M disodium hydrogen phosphate heptahydrate* dilute

to 100 ml with water, Adjusted to pH 6.8 and dilute to 1000 ml with *water*.

For Total PRP determination

Test solution (a). Pool the contents of two 10 dose per vials (5ml) and the content of 14 doses (0.5 ml per dose) of vaccine in a falcon tube of 15 ml after vigorous shaking of each dose for 30 seconds.

Test solution (b). Shake vigorously test solution (a) for 30 seconds. Take 1.0 ml of the suspension and pour it in a micro tube or acid hydrolysis, in duplicate and mark as TOT-S1 and TOT-S2.

For Free PRP content determination in the presence of adjuvant

Test solution (c). Shake vigorously the test solution (a) for 30 seconds. Transfer 4.0 ml of the suspension to a 10 ml-volumetric flask. add 50 µl of the buffer solution and dilute to volume with Test solution(a). Stopper the flask and invert gently several times. Leave the suspension under agitation for atleast 1 hour at room temperature.

NOTE — This treatment could be done one day before the centrifugation described below. In this case the sample is left at 5 ± 3°, over night.

Transfer 2.4 ml of the suspension to each of two 15 ml falcon tubes. Centrifuge both falcon tubes at 2300 rpm for 30 minutes at 10°. Collect the supernatant in two eppendrof tubes, marked as S1 and S2 respectively and both the supernatants (S1 and S2) through activated SPEC4 cartridges to separate free PRP from PRP.

For Free PRP content determination in the absence of adjuvant

Test solution (d). The test solution (a) for 30 seconds. Transfer 4.0 ml of the suspension to a 5-ml volumetric flask add 50 µl of *water* and dilute to volume with test solution (a). Stopper the flask and invert gently.

NOTE — The sample can be prepared the day before the hydrolysis. In this case the sample is left at 5 ± 3°, overnight.

Transfer 2.5 ml of the suspension to each of two 15 ml falcon tubes, marked as S1 and S2 respectively. Both the solutions are passed through activated SPEC4 cartridges to separate Free PRP from total PRP.

NOTE — C4S.P.E. Cartridge activation. Activate the cartridge as stated by manufacturer instructions (gravity method) or use a peristaltic pump (100m bar). Pass through the cartridge the following volumes, paying attention to never dry the packing: 3 ml of methanol then 10 ml of degassed water for washing.

Reference solution (a). Prepared by reconstituting the content of the one ampoule in 1.0 ml *water* to obtain a solution having concentration 0.4933 per cent w/v of PRP.

Reference solution (b). Dilute 100 µl of reference solution (a) to 25.0 ml with *water*.

Reference solution (c). A 0.015 per cent w/v solution of *ribitol RS* in water. Dilute 1.0 ml of the solution to 50.0 ml.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm,strongly cation exchange column consisted of monoporus resin (7.5µm) (such as CarboPac MA1),
- mobile phase: *0.58 M sodium hydroxide*,
- sampler temperature: 4 - 6°,
- flow rate: 0.4 ml per minute,
- detector. PAD,
- injection volume: 100 µl,
- run time: 40 minutes.

Separation of the Free PRP from the conjugate in the S1 and S2 Supernatants

By gravity or by the use of a peristaltic pump (50 m bar) pass through the cartridge the following volumes, paying attention to never dry the packing and collecting the permeate in a 2 ml volumetric flask: 1 ml of S1 or S2 Supernatants. 1 ml of sodium chloride 0.9 per cent. The final volume of permeate needs to be 2 ml. If necessary, additional amount of sodium chloride 0.9 per cent is passed through the cartridge up to reach the requested volume. Stopper the flask and invert gently several times. Take 1 mL from each permeate (Free-S1 or Free-S2) and poor it in the micro tube for acid hydrolysis.

Acid Hydrolysis / Depolymerization

To 1 ml of each Sample/Standard, add 50 µl of *6 M hydrochloric acid*. Hydrolyse samples and standards in a dry oven set at 100 ± 2° for 2 hours. Cool samples and standards at 5 ± 3° for 10 minutes. Add 400 µl of *1 M sodium hydroxide* to each samples and standards. Soon after, if it is not possible to start the analysis by HPAEC-PAD, store all Samples and Standard at 5 ± 3° until the next day. To determine the total saccharide in TOT-S 1 and TOT-S 2 samples, make up a dilution of the samples 1:20 with *0.07 M sodium hydroxide* (50 µl TOT-S + 950 µl *0.07 M sodium hydroxide*). This dilution takes into account that the total PRP content is generally in the range 8-12 µg /shd). Shake each tube with vortex for 5 seconds. Filter samples and standards on 0.22 µm before instrumental analysis.

Preparation of standard curves

The free and total polysaccharide content in the vaccine sample can be calculated *vs* two different calibration curves: the WHO PRP International Standard curve and the Ribitol

Reference Standard curve. The preparation of the two calibration curves is described.

Preparation of Standard Solutions for the PRP calibration curve

Directly arrange the specific volumes of working solution and water in the micro tubes for hydrolysis as described in the table below to obtain 1 ml.

Standard	Volume in µl of Water	Volume in µl of Reference solution (a)	Final Dilution of Reference solution (a)	Final concentration Reference solution (a) (µl/ml)
STD-PRP1	993.7	6.3	1:160	0.12
STD-PRP2	987.5	12.5	1:80	0.25
STD-PRP3*	975	25	1:40	0.49
STD-PRP4*	950	50	1:20	0.99
STD-PRP5	900	100	1:10	1.97

*These two standards are used as SST samples, when results are calculated by Ribitol Calibration Curve.

NOTE — Shake each tube with vortex, 5 seconds .Subject the standard samples to acid hydrolysis for depolymerization.

Preparation of Standard Solutions for the Ribitol calibration curve

Directly arrange the specific volumes of working solution and water in the microtubes for hydrolysis as described in the table below to obtain 1 ml.

Standard	Volume in µl of Reference solution (b) (3 µl/ml)	Volume in µl of Water	Final Concentration of Reference solution (b) (µl/ml)
STD-Ribitol1	25	975	0.075
STD-Ribitol2	50	950	0.15
STD-Ribitol3	150	850	0.45
STD-Ribitol4	250	750	0.75
STD-Ribitol5	350	650	1.05

NOTE — Shake each tube with vortex for 5 seconds Subject the standard samples to acid hydrolysis for depolymerization.

Calculation of PRP

Two calibration curves can be created by the software.

By using the PRP Calibration curve, the results are obtained in PRP concentration and need to be corrected only for the dilution factor. By using the ribitol Calibration curve the results are expressed as ribitol concentration of the analysed sample. To obtain the vaccine PRP content, it is necessary to multiply first for the dilution factor and after, as ribitol accounts for 41.3 percent of the PRP dry weight, also for the conversion factor of 2.42.

Determination of Total PRP

Total PRP is derived from the mean value of TOT-S1 and TOT-S2 and should be reported as ig/shd, quoted to 2 decimals.

Determination of the percent of Free PRP

Free PRP content is derived from the mean value of S1 and S2 and should be reported as ig/shd (quoted to 2 decimals) and in percentage.

The percentage is calculated by the formula

$$\text{Per cent Free PRP} = \frac{\text{Free PRP}}{\text{TOT - PRP}} \times 100$$

Acceptance criteria

Test validity

- Chromatograms of blank, *water* and *sodium chloride* 0.9 per cent are free from any peak in the elution range of ribitol;
- r^2 is ≥ 0.990 ;
- In-house reference CP obtained from the mean of two values is in the range 90-110percent of the theoretical value of 0.3 ig/ml;
- The two PRP SST values are in the range 80-120 percent of the concentration of the Working PRP solution.

Number of theoretical plates not less than 40percent of those determined at qualification. Calculate them considering the mean value of theoretical plates of CP1 and CP2.

Symmetry Factor 0.8 -1.5 . Calculate it considering the mean value of symmetry factor of CP1 and CP2.

Vaccine samples

- CV percent ≤ 10 percent for the two determinations of the Total PRP content;
- CV percent ≤ 10 percent for the two determinations of the Free PRP content;
- The total PRP concentration is not be less than 80.0 percent and not more than 120.0 per cent of the amount of the stated on the label.

The amount of Free PRP is not more than that approved for the particular product.

2.7.16. Adjuvants for Vaccines

The word "adjuvant" comes from the Latin word *adjuvare*, which means "to help or aid". Adjuvant is a substance or combination of substances used in conjunction with a vaccine antigen to enhance (for example, increase, accelerate, prolong and/or possibly target) or modulate a specific immune response to the vaccine antigen in order to enhance the clinical effectiveness of the vaccine. Adjuvants are added to vaccine formulations to increase the immunogenicity of antigens and induce protection against infection. Presently different kinds of vaccines are in use in humans, (i) live-attenuated vaccines, (ii) inactivated vaccines and (iii) conjugate vaccine, (iv) purified subunit vaccines, (v) Toxoids. Vaccines prepared from live-attenuated or inactivated pathogens, or via conjugation of bacterial capsular polysaccharides to carrier proteins, generally elicit robust protective immune responses on their own and do not require adjuvants. By contrast, protein-based vaccines (purified native or recombinant) typically have low immunogenicity and require formulation with adjuvants to induce long-lasting protective immune responses. The use of a suitable adjuvant in a given vaccine formulation, where required, serves to selectively trigger innate immunity and/or adaptive immune pathways in order to induce a specific protective response. While the key underlying mechanisms responsible for vaccine adjuvant function are not fully understood, advancements in technology and knowledge about the interaction of immune system with pathogens holds the promise of rational design of vaccine formulations including the adjuvant components. The requirement for an adjuvant, as well as the type of adjuvant and specific formulation design, cannot be predicted *a priori* and therefore must be established and justified on a case by case basis through clinical experience with specific vaccine formulations.

Adjuvants are generally divided into three main types. The first main type comprises of those that increase oraugment the delivery of antigen to antigen-presenting cells and/or the lymph nodes, consequently improving the immune response. The aluminium based adjuvants, the oil-in-water emulsions and liposomes are some of the examples. The second type includes immunostimulators or immunopotentiators, which act mainly by receptor-mediated signalling pathways that help in modulating the quality of the immune response. Examples are aluminium based adjuvants, Monophosphoryl lipid A (MPL) that activate Toll-like receptor 4 (TLR-4), QS21, CpG that activate TLR-9, cytokines, etc. The third category includes the combination of the first two types and are termed as combinations or "adjuvant systems". AS04 is one of the examples, which is made up of MPL adsorbed onto aluminium-salt and is used as an adjuvant in hepatitis vaccine and in the Human Papilloma Virus (HPV) vaccine. Other examples of adjuvant systems include AS03,containing α-tocopherol and squalenein oil-in-water emulsion (o/w)and AS01 adjuvant, which is a combination of MPL and QS21 in liposomes.

Adjuvants for Human Use

Aluminium salts or aluminium based adjuvants

Aluminium saltsare the most commonly used adjuvantsin human vaccines and have been used in vaccination for over 80 years.

Aluminium salt adjuvants have complex,heterogeneous physical structures and the antigen is adsorbed ontothe adjuvant through hydrophobic, electrostatic interactions and ligand exchange interactions between antigen and the aluminium salt. Different aluminium salts are contained in numerous licensed vaccineseg., aluminium hydroxide, aluminium phosphate and aluminium hydroxyphosphate. Generally, aluminium hydroxide ispositively charged at a physiological pH of 7.4 and binds acidic proteins. On the other hand, aluminium phosphate is negatively charged and therefore binds basic proteins. However, it is required to evaluate the optimum conditions with adjuvants, when combination vaccine are adsorbed onto aluminium adjuvants, where drug substances would have different physico-chemical properties.

"Depot/repository effect" is one of the most well-known mechanism of action of alum. Alum facilitates humoral immunity via Th2 type immune responses in mice (IgG1, IgE, IL-4, IL-5 and eosinophil). The advantages of using alum as an adjuvant include the stabilization of antigen and enhancement of high and long-lasting antibody titre. Limitations include alum induce poor Th1 type immunity and cytotoxic T cell response along with the limitation of sterilization by filtration or being frozen or lyophilized for the vaccines containing alum adjuvants.

Oil-in-water (o/w) emulsions

Emulsions are two-phase systems comprising a minimum of two immiscible liquids, combined with a surfactant for stabilizing. The key benefits of using emulsions are antigen dose sparing and enhancement of antibody titre. For example: MF59 and adjuvant system 03 (AS03). MF59 is an oil-in-water emulsion (o/w) containing squalene as the dispersed phase and two non-ionic surfactants- Tween 80 and Span 85.AS03 is a combination of adjuvants based on α-tocopherol (vitamin E) and squalene in an oil-in-water emulsion (o/w).

Both MF59 and AS03 are squalene based oil-in-water emulsions. MF59 recruits monocytes and macrophages into injection sites by the induction of local chemokine secretion. It can also increase antigen uptake by dendritic cells (DCs) and activate CD4 T cells. Consequently, MF59 generates high antibody titres with balanced IgG1:IgG2a responsesin mice.

AS03 triggers transient production of cytokines at the injection site and in the draining lymph nodes (dLNs). α-tocopherol in AS03 modulates the expression of some cytokines such as CCL2, CCL3, IL-6, CSF3 and CXCL, increases the antigen loading in monocytes and the recruitment of granulocytes in the dLNs.

Liposomes

In the following Immune stimulating complexes(ISCOM) and Virosomes are included for information.

Immune stimulating complexes (ISCOM)

ISCOMs are another potential lipid-based adjuvants. They are spherical and ring-like structures that are spontaneously produced upon mixing antigens with saponin, cholesterol and phospholipid. The compound QS-21, a potent immune stimulatory saponin configured with ISCOM adjuvant is being considered as a potent vaccine adjuvant system reducing the dose and hence the toxicity of QS-21. Main benefits of these adjuvants are induction of high and long-lasting antibody titre, induction of balanced Th1 and Th2 type immunity, and initiation of CMI including cytotoxic T cell response.

Virosomes

A virosome is a reconstituted viral envelope comprised of membrane lipids and viral glycoproteins, butlack viral genetic information. Some vaccines like influenza virus vaccine and hepatitis A virus vaccine utilize virosomes derived from influenza virus represented by Immunopotentiating reconstituted influenza virosomes (IRIV) harbouring the influenza heamagglutinin (HA) protein. Since virosomal adjuvants present antigen through both major histocompatability complex (MHC) I and MHC II, virosomes can induce both humoral immunity and cell mediated immunity (CMI). Benefits of usingvirosomes in vaccines are: (i) high quality and long-lasting antibody responses, (ii) conformational stabilization of antigen, (iii) protection of antigen from degradation, (iv)excellent safety profile, (v) suitability to specific populations such as infants, immune-compromised patients, and the elderly.

TLR agonists

TLRs provide a bridge between innate and adaptive immunity. A new category of effective vaccine adjuvant is based on the TLR pathway. TLR 9 is one of the more advanced adjuvant candidates among TLR agonists. Unmethylated CpG oligodeoxynucleotides (ODN) area type of TLR 9 agonist (For example immunostimulatory sequences CpG 7909) that enhance antigen-specific immune responses and induce proinflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ. CpG ODN is an example of immunostimulatory sequences (ISS).

Monophosphoryl lipid A (MPL)

On the basis of use of TLR agonists, potential progress has been made in the formulation of efficient adjuvants and some of these adjuvants have already been licensed for human vaccines. MPL, a TLR4 agonist, is obtained by chemical detoxification of the parent lipopolysaccharide (LPS) of *Salmonella Minnesota* R595 strain. MPL enhances the production of pro-inflammatory cytokines such as IL-2 and IFN-γ, and consequently generates Th1 immune responses. For example: AS04 is composed of MPL adsorbed to aluminium salts. Use of AS04 adjuvant in human vaccines effectively stimulates robust Th1-type immune responses by promoting IL-2 and IFN-γ production, which cannot be achieved by using alum alone. The antigen is co-localized with AS04 in lymph nodes to elicit an adjuvant effect on antigen presenting cells.

$AS01_B$ is an adjuvant suspension used with the antigen component of recombinant zoster vaccine, which recommended for persons aged 50 years or older. $AS01_B$ is made of up of monophosphoryl lipid A (MPL), an immune-boosting substance isolated from the surface of bacteria, and QS-21, a natural compound extracted from the Chilean soapbark tree (Quillajasaponaria Molina).

Regulatory Pathways and Testing Aspects for Adjuvants

Adjuvants itself are not licensed but a specific adjuvant/ antigen formulation is licensed. Therefore, an adjuvant is developed strictly according to the antigen present in the specific formulation.Vaccine regulatory procedures for licensing or lot release of adjuvants are not harmonized globally. Hence for harmonisation to some extent, it is recommended to follow various applicable International guidelines such as WHO, ICH etc and national regulatory requirements. An international guideline 'Guideline on adjuvants in vaccines for human use' clarify the difference between immunomodulators and adjuvants. It has also been stated that adjuvants should be a part of the formulation to be administered, whereas compounds that are given separately and/or at a different time point are called immunomodulators. An adjuvant is intended to help the immune response by a confined, synchronized and concomitant action with the antigen. Since vaccines are administered to healthy individuals includes infants and children and there may be safety related issues with novel adjuvants, there is a need of substantial toxicological studies in animals for adjuvants and adjuvanted vaccines, including local reactogenicity and repeated dose toxicity studies. Safety of any adjuvant must be adequately demonstrated by means of appropriately designed preclinical and clinical safety studies. Various national and international guidelines shall be referred for non-clinical evaluation of vaccine adjuvants and adjuvanted vaccines.

Performance characteristics/Quality specifications of adjuvants

Adjuvants are included in the formulation of a vaccine to potentiate and/or modulate the immune response to the antigen(s). Suitable characterisation and quality control of the adjuvant(s), alone and in combination with the antigen(s), is essential for consistent production. Existing tests methods maynot be sufficient to ensure the consistency in adjuvant manufacturing. Eg. Aluminium or other adjuvant systems. Vaccine manufacturer should identify/integrate the additional tests to ensure consistency.

Quality specifications are established for each adjuvant, alone and in combination with the antigen(s).

Adsorbents as adjuvants. Vaccines may be adsorbed on aluminium hydroxide, aluminium phosphate, calcium phosphate or other suitable adsorbents. The adsorbents are prepared in special conditions that confer the appropriate physical form and adsorptive properties.

In situadjuvants. Where an adsorbent is used as an adjuvant and is generated *in situ* during production of the vaccine, quality specifications should be established for each of ingredients used in the production of the adsorbent and for the generated adsorbent in the vaccine.

Quality specifications are intended to control, in particular:

- qualitative and quantitative chemical composition;
- physical form and associated adsorptive properties, where relevant, and particularly where the adjuvant will be present as an adsorbent;
- interaction between adjuvant and antigen;
- purity, including bacterial endotoxin content and microbiological quality;
- any other parameters identified as being critical for functionality such as particle size and distribution to be is established during development studies.
- stability of each adjuvant, alone and in combination with the antigen(s), particularly for critical parameters is established during development studies.

If the vaccine contains an adjuvant, the amount is determined and shown to be within acceptable limits with respect to the expected amount unless otherwise stated.

Table – Adjuvants Approved For Vaccines for Human Use

Adjuvant Name	Class	Vaccine
Aluminium hydroxide gel or hydrated Aluminium phosphate gel	Aluminium salts/ mineral salts/ Alum	• DPT group of vaccines • Hepatitis B Vaccine (rDNA) • Inactivated Hepatitis A Vaccine (Adsorbed) • Human Papillomavirus Vaccine (rDNA) • Rabies vaccine • Pneumococcal vaccines • Meningococcal vaccines • Anthrax • HIB • Japanese encephalitis
Adjuvant system 04 (AS04)	MPL (3-*O*–Desacyl-4'-Monophosphoryl Lipid A) and Aluminium phosphate Alum adsorbed TLR4 agonist	• Human Papillomavirus vaccine • Hepatitis B Vaccine
MF 59	Oil in water emulsion	• Influanza vaccine
AS03	Oil in water emulsion	• Pandemic H1N1 Influanza vaccine
$AS01_B$	Monophosphoryl lipid A (MPL) and QS-21, a natural compound extracted from the Chilean soapbark tree, combined in a liposomal formulation	• Herpes zoster vaccine
CpG 1018	TLR 9 agonists	• HEPLISAV-B

2.8. TESTS ON BLOOD AND BLOOD-RELATED PRODUCTS

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2.8.1. Nucleic Acid Amplification Techniques

Nucleic acid amplification techniques are based on two different approaches:

1. Amplification of a target nucleic acid sequence using polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification;
2. Amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method. In this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

Scope. This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

Principle of the method. PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, two synthetic oligonucleotide primers of opposite polarity, anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions which form as a result of specific base pairing between the primers and the complementary DNA sequence, border the DNA segment to be amplified and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of (a) heat denaturation of the nucleic acid (target sequence) into 2 single strands; (b) specific annealing of the primers to the target sequence under suitable reaction conditions; (c) extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

Multiplex PCR assays use several primer pairs designed for simultaneous amplification of different targets in one reaction.

Test material. Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimize degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

Test method

Prevention of contamination. The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:

1. Master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.);
2. Pre-PCR (area where reagents, samples and controls are handled);
3. PCR amplification (amplified material is handled in a closed system);
4. Post-PCR detection (the only area where the amplified material is handled in an open system).

Sample preparation. When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under Internal control must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

Amplification. PCR amplification of the target sequence is conducted under defined cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as: (a) the length and base composition of primer and target sequences; (b) the type of DNA polymerase, buffer composition and reaction volume used for the amplification; (c) the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

Detection. The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Detection and characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, liquid chromatography). Detection and characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Detection and characterisation by chemical modification may be achieved by incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent radioisotopic or immuno-enzyme-coupled detection.

Evaluation and interpretation of results. A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result. As soon as a measurable target threshold is defined, a quantitative test system is required.

Quality assurance

Validation of the PCR assay system. The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the *ICH guidelines* (topic Q2B) Validation of Analytical Method: Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences for which the test system will be used are indispensable for validation of a PCR test.

Determination of the positive cut-off point. During validation of qualitative tests, the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample which can be detected in 95.0 per cent of test runs. The positive cut-off point depends on inter-related factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

Quantitative assay systems. For a quantitative assay, the following parameters are determined during validation: accuracy, precision, specificity, quantitation limit, linearity, range and robustness.

Quality control of reagents. All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are the crucial component of the PCR assay and as such their design, purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

Run controls

External controls. In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR assay:

Positive control. This contains a defined number of target-sequence copies, the number being close to the positive cut-off value, and determined individually for each assay system and indicated as a multiple of the positive cut-off value of the assay system;

Negative control. A sample of a suitable matrix already proven to be free of the target sequences.

Internal control. Internal control is defined as nucleic acid sequences containing, unless otherwise prescribed, the primer binding sites. Internal control must be amplified with defined efficacy, and the amplicons must be clearly discernible. Internal control must be of the same type of nucleic acid (DNA/RNA) as the material under test. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

Threshold control. The threshold control for quantitative assays is a test sample with the analyte at a concentration which is defined as the threshold not to be exceeded. It contains the analyte suitably calibrated in IU and is analysed in parallel in each run of a quantitative assay.

External quality assessment. Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

The following section is published for information.

Validation of Nucleic Acid Amplification Techniques (NAT) for the Detection of Hepatitis C Virus (HCV) RNA in Plasma Pools: Guidelines

Scope. The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the National or International Guidelines. These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, two characteristics regarded as most important for validation of the analytical procedure are specificity and detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part of or the complete analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross contamination).

Specificity. Specificity is the ability to unequivocally assess nucleic acid in the presence of components which may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

While designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive.

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes should be detected at a suitable level.

Detection limit. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to two, either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point is the minimum number of target sequences per volume sample which can be detected in 95.0 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency and can result in different 95.0 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus reference preparation*, which has been calibrated against the WHO HCV International Standard 96/790, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using log dilutions of the plasma pool sample) should be done in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using a dilution factor of 0.5 log or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA which can be detected in 95.0 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

Robustness. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95.0 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods which use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV specific antibodies, should be tested and found positive.

Cross contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least 10^2 times the 95.0 per cent cut-off value or at least 10^4 IU/ml).

Quality assurance. For biological tests such as NAT, specific problems may arise which may influence both the validation and interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover: (a) the mode of sampling (type of container, etc.); (b) the preparation of mini-pools (where appropriate); (c) the conditions of storage before analysis; (d) the exact description of the test conditions, including precautions taken to prevent cross contamination or destruction of the viral RNA, reagents and reference preparations used; (e) the exact description of the apparatus used; (f) the detailed formula for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus reference preparation* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard 96/790) can be considered a satisfactory system suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification. An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. Confirmation of analytical procedure performance after change of critical equipment (e.g. thermocyclers) should be documented by conducting a parallel test on 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95.0 per cent cut-off value. All results should be positive.

Operator qualification. An appropriate qualification programme should be implemented for each operator involved in the testing. To confirm successful training each operator should test at least 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95.0 per cent cut-off value. This test (8 replicate samples) should be repeated twice on two separate days, i.e. a total of 24 tests performed on three different days. All results should be positive.

2.8.2. Assay of Human Anti-D Immunoglobulin

Method A

The potency of human anti-D immunoglobulin is determined by comparing the quantity necessary to produce agglutination of D-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity contained in a stated amount of the International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Human anti-D immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Use pooled D-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not fewer than 4 group 0 R_1R_1 donors. To a suitable volume of the cells, previously washed 3 times with a 0.9 per cent w/v solution of *sodium chloride*, add an equal volume of *bromelains solution*, allow to stand at 37° for 10 minutes, centrifuge, remove the supernatant liquid and wash 3 times with a 0.9 per cent w/v solution of *sodium chloride*. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 30.0 per cent w/v solution of *bovine albumin* and 45 volumes of a 0.9 per cent w/v solution of *sodium chloride*. Stand the resulting suspension in iced water, stirring continuously.

Using a calibrated automated dilutor, prepare suitable dilutions of the preparation under examination and of the reference preparation using as diluent a solution containing 0.5 per cent

w/v of *bovine albumin* and 0.9 per cent w/v of *sodium chloride*.

Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils, at 15°. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 ml per min and a 0.3 per cent w/v solution of *methylcellulose 450* at a rate of 0.05 ml per min. Introduce the dilutions of the preparation under examination and the reference preparation at a rate of 0.1 ml per min for 2 minutes, followed by the *diluent solution at* a rate of 0.1 ml per min for 4 minutes before the next dilution is introduced. Introduce air at a rate of 0.6 ml per min. Incubate at 37° for 18 minutes and then disperse the rouleaux by introducing at a rate of 1.6 ml per min a 0.9 per cent w/v solution of *sodium chloride* containing a suitable wetting agent (for example, *polysorbate 20* at a final concentration of 0.02 per cent w/v to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 ml per min and then at 0.6 ml per min. Lyse the unagglutinated red blood cells with a solution containing 0.5 per cent w/v of *octoxinol 10*, 0.02 per cent w/v of *potassium ferricyanide*, 0.1 per cent w/v of *sodium hydrogen carbonate* and 0.005 per cent w/v of *potassium cyanide* at a rate of 2.5 ml per min. A ten-minute delay coil is introduced to allow for conversion of the haemoglobin. Continuously record the absorbance (2.4.7) of the haemolysate at a wavelength between 540 to 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between concentration and the resultant change in absorbance (AA). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation under examination.

Calculate the potency of the preparation under examination using the usual statistical methods (5.7).

Method B

The potency of human anti-D immunoglobulin is determined by competitive enzyme-linked immunoassay on erythrocyte-coated microtitre plates. The method is based on the competitive binding between a polyclonal anti-D immunoglobulin preparation and a biotinylated monoclonal anti-D antibody directed against a D-antigen specific epitope. The activity of the preparation under examination is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International reference preparation is stated by the World Health Organisation. Human anti-D immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard and Intended for use in the assay of human anti-D immunoglobulin.

Materials. Reagents not specified are of analytical grade.

PBS (Phosphate-buffered saline). Dissolve 8.0 g of *sodium chloride*, 0.76 g of *anhydrous disodium hydrogen phosphate*, 0.2 g of *potassium chloride*, 0.2 g of *potassium dihydrogen phosphate* and 0.2 g of *sodium azide in water* and dilute to 1000 ml with the same solvent.

TBS (Tris-buffered saline). Dissolve 8.0 g *of sodium chloride* and 0.6 g of *tris (hydroxymethyl) aminomethane in water*. Adjust to pH 7.2 with *1 M hydrochloric acid* and dilute to 1000 ml with the same solvent.

Papain solution. Prepare a solution by stirring 1 g *of papain* at 37° for 30 minutes in 10 ml of *0.067 M phosphate buffer solution pH 5.4*, centrifuge at 10, 000 g for 5 minutes and filter through a membrane with a pore size of 0.22 µm. To activate, combine 1 ml of the filtrate with 1 ml of a 4.844 per cent w/v solution *of L-cysteine* and 1 ml of a 0.372 per cent w/v solution *of sodium edetate* and dilute to 10.0 ml with *0.067 M phosphate buffer solution pH 5.4*. Freeze in aliquots at - 20° or below.

Red blood cells. Use pooled D-positive red blood cells obtained from not fewer than 3 group O R_2R_2 donors. Wash the cells 4 times with *PBS*. Centrifuge the cells at 1800 *g* for 5 minutes, mix a suitable volume of pre-warmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume suitable) and incubate at 37° for 10 minutes. Wash the cells 4 times with *PBS*. Store at 4° in an appropriate stabiliser for up to 1 week.

Biotinylated Brad-5. Use according to instructions.

Alkaline phosphatase-conjugated avidin/streptavidin reagent. Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

Substrate solution. Use *para-nitrophenyl phosphate* according to instructions.

Cell fixation buffer. Dissolve 18.02 g of *glucose*, 4.09 g of *sodium chloride*, 1.24 g of *boric acid*, 10.29 g *of sodium citrate* and 0.74 g of *sodium edetate* in *water*. Adjust to pH 7.2 to 7.3 using 1 *M sodium hydroxide* or *1 M hydrochloric acid*, and dilute to 1000 ml with *water* and store at 4°.

Glutaraldehyde solution. Immediately before use, add 90 µl of a 25.0 per cent w/v solution of *glutaraldehyde* to 24 ml of cold *PBS*.

Microtitre plates. Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U or V-bottomed polystyrene or poly (vinyl chloride) plates.

Method. Prepare a 0.1 per cent v/v suspension of papain-treated red blood cells in cold cell fixation buffer. Pipette 50 µl into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 g for 3 minutes, preferably at 4°. Without removing the supernatant, gently add 100 µl of glutaraldehyde solution to each well and leave for 10 minutes.

Drain the wells by quickly inverting the plate and wash 3 times with 250 to 300 pl of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4° after draining off the PBS and adding 100 µl of cell fixation buffer per well and sealing with plastic film. Plates can be stored at 4° for up to 1 month.

Test solutions. For freeze-dried preparations, reconstitute as stated on the label. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU per ml in PBS containing 1.0 per cent w/v of bovine albumin. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU per ml in PBS containing 1.0 per cent w/v of bovine albumin.

Using U or V-bottomed microtitre plates, add 35 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 µl of biotinylated Brad-5 at 250 ng per ml.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel. Add 250 µl of PBS containing 2.0 per cent w/v of *bovine albumin* and leave at room temperature for 30 minutes.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 µl from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 µl of PBS containing 1.0 per cent w/v of bovine albumin as negative control. Seal the plate with plastic film and incubate at room temperature for 1 hour.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250 to 300 µl of TBS.

Dilute the alkaline phosphatase-conjugated avidin/streptavidin reagent in TBS containing 1.0 per cent w/v of bovine albumin and add 50 to each well. Incubate for 30 minutes at room temperature.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250 to 300 pi of TBS.

Add 100 µl of substrate solution to each of the, wells incubate at room temperature for 10 minutes in the dark. To stop the reaction, add 50 pi of 3 M sodium hydroxide to each of the wells.

Measure the absorbances at 405 nm and substract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation under examination by the usual statistical methods (5.7).

Method C

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation under examination is compared with are reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Human anti-D immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Materials. Reagents used are of analytical grade.

PBS. Dissolve 8.0 g of sodium chloride, 0.76 g of disodium hydrogen phosphate, 0.2 g of potassium chloride and 0.2 g of potassium dihydrogen phosphate in water and dilute to 1000 ml with the same solvent.

PBS-BSA solution. PBS containing 1.0 per cent w/v of bovine albumin.

Red blood cells. Use D-positive red blood cells obtained from a group 0 R_1 R_1, donor within 2 weeks of collection. Store if necessary in an appropriate stabiliser at 4°. Wash the cells at least twice with PBS-BSA solution and prepare a suspension containing 1 x 10^4 cells per microlitre but not more than 5 x 10^4 cells per microlitre in PBS- BSA solution.

Use D-negative red blood cells obtained from a group 0 R_1R_1donor and prepared similarly.

Secondary antibody. Use a suitable fluorescent dye conjugated anti-IgG antibody-fragment specific for human IgG or parts of it. Store and use according to the manufacturer's instructions.

Microtitres plates. Use flat-bottomed plates without surface treatment for enzyme immunoassays.

Method

Test solutions. For freeze-dried preparations, reconstitute as stated on the label. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting with a concentration in the range of 1.2-0.15 IU per ml using PBS-BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting with a concentration in the range of 1.2 - 0.15 IU per ml using PBS-BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µl of the D-positive red blood cells into each well of a microtitre plate. Add 50 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µl of PBS-BSA solution as negative control. Distribute 50 µl of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µl of the lowest dilution of the test preparation. To monitor spurious reactions distribute 50 µl of the D-positive red blood cells into 4 wells of the same Microtitre plate and add 50 µl of PBS-BSA solution. Seal with plastic film and incubate at 37° for 40 minutes.

Centrifuge the plates at 50 g for 3 minutes, discard the supernatant and wash the cells with 200 to 250 µl of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 g for 3 minutes, discard the supernatant and add 50 µl of the *secondary antibody diluted* with PBS-BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 minutes.

Centrifuge the plates at 50 g for 3 minutes, discard the supernatant and wash the cells with 200 to 250 µl of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 g for 3 minutes, resuspend the cells into 200 to 250 µl of PBS. Transfer the cell suspension into a tube suitable for the flow cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate.

Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10, 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose response curve to estimate the potency of the preparation under examination by the usual statistical methods (5.7).

Method D

By Agglutination. Determine the assay by Anti-D agglutination Titration Method as follows.

Preparation of 4 per cent red blood cell suspension. Prepare 4 per cent red blood cell suspension from freshly collected (not less than 3) O+ ve red blood cells (Rh D +ve of any phenotype) in Normal Saline.

Preparation of standard. Reconstitute the reference standard and further dilute with 10 per cent w/v. Bovine serum Albumin prepared in 0.9 per cent w/v Sodium chloride to a concentration of about 1.14 µg per ml. Prepare serial dilutions of standard starting from 1.14 µg per ml in a range of 1:2 to 1:512.

Preparation of sample. Dilute the substance under examination to a concentration of 150 mcg per ml in water for injection if required. Prepare 1:1000 dilution of sample 150 mcg per ml) using 10 per cent w/v Bovine Serum Albumin prepared

Table 1

	1	2	3	4	5	6	7	8	9
Dilutions	2000	3000	4000	5000	6000	7000	8000	9000	10000
1.1000 dilution	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
10 per cent BSA	50 µl	100 µl	150 µl	200 µl	250 µl	300 µl	350 µl	400 µl	450 µl
	10	11	12	13	14	15	16	17	18
Dilutions	11000	12000	13000	14000	15000	16000	17000	18000	20000
1.1000 dilution	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
10 per cent BSA	500 µl	550µl	600 µl	650 µl	700 µl	750 µl	800 µl	850 µl	950 µl
	19	20	21	22	23	24	25	26	27
Dilutions	22000	24000	26000	28000	29000	30000	31000	32000	33000
1.1000 dilution	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
10 per cent BSA	1050 µl	1150 µl	1250 µl	1350 µl	1400 µl	1450 µl	1500 µl	1550 µl	1600 µl
	28	29	30	31	32	33	34		
Dilutions	**34000**	**35000**	**36000**	**37000**	**38000**	**39000**	**40000**		
1.1000 dilution	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl		
10 per cent BSA	1650 µl	1700 µl	1750 µl	1800 µl	1850 µl	1900 µl	1950 µl		

in 0.9 per cent w/v Sodium Chloride (Solution A). Prepare further dilutions as shown in Table 1 by using Solution A (10 per cent w/v Bovine Serum Albumin prepared in 0.9 per cent w/v Sodium Chloride).

Transfer 0.1 ml of each standard dilution and sample dilutions into serological test tubes, add 0.1 ml of 4 per cent cell suspension to each tube of standard and sample dilution.

Incubate all the tubes at 37° ± for 90 minutes. After incubation wash the cells 4 times with saline by centrifuging at 4000 - 5000 rpm for 5 minutes. Decant the supernatant and add 100 µl of Polyspecific Anti-Human Globulin (Polyspecific AHG) to the packed cells and incubate at a temperature between 20 to 25° for 30 minutes.

After completion of incubation, centrifuge all the tubes at 1000 rpm for 1minute and observe for agglutination macroscopically by re-suspending the red cell by agitation over a light source and under white background. Observe the agglutination for all the tubes and note the maximum serial dilution tube showing agglutination. This particular dilution factor is the end point of the titration.

Calculation. For eg.: Calculate the potency of the product using following formula:

$$\text{Potency} = \frac{\text{Maximum dilution at agglutination observed} \times \text{Dilution factor} \times 1.14}{256}$$

$$= \ldots\ldots \text{ mcg per ml}$$

NOTE — **Standard** 1.14 µgm per ml is equivalent to titer of 1:256 (Variable, based on the value of Reference standard used and titre obtained).

The activity of the preparation under examination is compared with a reference preparation calibrated in international units. The estimated potency is not less than 90% of the stated value.

2.8.3. Activated Coagulation Factors

Where applicable, determine the amount of heparin present (2.8.9) and neutralise the heparin, for example by addition of *protamine sulphate* (10 µg of protamine sulphate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation under examintion using *tris(hydroxymethyl) aminomethane buffer solution pH 7.5*. Place a series of polystyrene tubes in a water-bath at 37° and add to each tube 0.1 ml of *platelet-poor plasma* and 0.1 ml of a suitable dilution of a phospholipid preparation to act as a platelet substitute. Allow to stand for 60 seconds. Add to each tube either 0.1 ml of 1 of the dilutions or 0.1 ml of the *buffer solution* (control tube). To each tube add immediately 0.1 ml of a 0.37 per cent w/v solution of *calcium chloride* previously heated to 37°, and measure, within 30 minutes of preparing the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 to 350 seconds.

2.8.4. Assay of Human Coagulation Factor II

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor II activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human blood coagulation factor II. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

Reagents

Viper venom specific factor II activator (Ecarin). A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4° and use within 1 month.

Factor IIa chromogenic substrate. Specific chromogenic substrate for factor IIa such as: *H*-D-*phenylalanyl*-L-*pipecolyl*-L-*arginine-4-nitroanilide dihydrochloride, 4-toluenesulphonyl-glycyl-prolyl*-L-*arginine-4-nitroanilide, H*-D-*cyclohexylglycyl-α-aminobutyryl*-L-*arginine-4-nitroanilide, D-cyclohexylglycyl*-L-*alanyl*-L-*arginine-4-nitroanilide diacetate*. Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 0.606 per cent w/v of *tris(hydroxymethyl)aminomethane*, 1.753 per cent w/v of *sodium chloride*, 0.279 per cent w/v of *(ethylenedinitrilo) tetra-acetic acid* and 0.1 per cent w/v of *bovine albumin* or *human albumin*. Adjust to pH 8.4 if necessary, using *hydrochloric acid*.

Method

Test solution. Dilute the preparation under examination with *dilution buffer* to obtain a solution containing 0.015 IU of

factor II per ml. Prepare at least 3 further dilutions in *dilution buffer*.

Reference solution. Dilute the reference preparation with *dilution buffer* to obtain a solution containing 0.015 IU of factor II per ml. Prepare at least 3 further dilutions in *dilution buffer*.

Warm all solutions to 37° in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37°, add 25 µl of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µl of *dilution buffer*, then 25 µl of *ecarin* and incubate for exactly 2 minutes. To each well add 25 µl of *factor IIa chromogenic substrate*.

Read the rate of change of absorbance (2.4.7) at 405 nm continuously over a period of 3 minutes and obtain the mean rate of change of absorbance (ΔA per minute). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 seconds, plot the absorbances against time on a linear graph and calculate ΔA per minute as the slope of the line. From the ΔA per minute values of each individual dilution of standard and test preparations, calculate the potency of the preparation under examination and check the validity of the assay by the usual statistical methods (5.7).

2.8.5. Assay of Human Coagulation Factor VII

Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VII activity of a stated amount of the International Standard which consists of freeze-dried plasma. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of 2 consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ion, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarised by the following scheme Figure 2.

Step 1

$$\text{Factor VII} \xrightarrow{\text{Tissue factor} + Ca^{++}} \text{Factor VIIa}$$

$$\text{Factor X} = \xrightarrow{\text{Factor VIIa} + Ca^{++} + \text{Tissue factor/Phospholipid}} \text{Factor Xa}$$

Step 2

$$\text{Chromogenic substrate} \xrightarrow{\text{Factor Xa}} \text{Peptide} + \text{Chromophore}$$

Figure 1. Schematic representation of the assay of human coagulation factor VII

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

Reagents. The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipid as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10 nmol per litre to 350 nmol per litre, preferably 14 nmol per litre to 70 nmol per litre. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipid component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of Ca^{2+} is 15 mmol per litre to 25 mmol per litre. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3 to 8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water* and used at a final concentration of 0.2 to 2 mmol per litre. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

Assay

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation under examination by adding the appropriate quantity of *water*; use within 1 hour. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 to 2.0 IU of factor VII per ml.

Prepare further dilutions of reference and test preparations using an *isotonic non-chelating buffer containing 1.0 per cent of bovine or human albumin*, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicate. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU per ml.

Prepare a control solution that includes all components except factor VII.

Prepare all dilutions in plastic tubes and use within 1 hour.

Step 1. Mix dilutions of the factor VII reference preparation and the preparation under examination with an appropriate volume of the *prewarmed coagulation factor reagent* or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37°. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the Reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 to 5 minutes, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

Step 2. Terminate the activation by the addition of a *prewarmed reagent containing a chromogenic substrate*. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by the addition of a suitable reagent, such as *acetic acid* (50.0 per cent w/v $C_2H_4O_2$) or a *citrate solution* (1 mol per litre) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 to 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (5.7).

2.8.6. Assay of Human Coagulation Factor VIII

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipid. The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VIII activity of a stated amount of the International Standard, which consists of a freeze-dried human coagulation factor VIII concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human coagulation factor VIII reference preparation is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of 2 consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme (Figure 2).

Step 1

$$\text{Factor X} \xrightarrow{\text{(Activated) Factor VIII, Factor IXa, phospholipid, } Ca^{++}} \text{Factor Xa}$$

Step 2

$$\text{Chromogenic substrate} \xrightarrow{\text{Factor Xa}} \text{Peptide} + \text{Chromophore}$$

Figure 2.

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the International Standard for human blood coagulation factor VIII concentrate as the standard, that the results obtained do not differ significantly.

It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa

generation in order to determine the time taken to reach 50.0 per cent of the maximal factor Xa generation.

Reagents. The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50.0 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay. Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least 2 separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The 2nd step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between 3 to 5 amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

Assay

Reconstitute the entire contents of 1 ampoule of the reference preparation and of the preparation under examination; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5 to 2.0 IU per ml.

The prediluent consists of haemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma. The prediluted materials must be stable beyond the time required for the assay.

Prepare further dilutions of the reference and test preparations using a non-chelating, appropriately buffered solution *tris(hydroxymethyl)aminomethane* or *imidazole*, containing 1.0 per cent of *human or bovine albumin*. Prepare at least 2 dilution series of at least 3 further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU per ml, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.

Step 1. Mix prewarmed dilutions of the factor VIII reference preparation and of the preparation under examination with an appropriate volume of the *prewarmed coagulation factor reagent* or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37°. Allow the activation of factor X to proceed for a suitable time, terminating the reaction (step 2) when the factor Xa concentration has reached approximately 50.0 per cent of the maximal (plateau) level. Appropriate activation times are usually between 2 to 5 minutes.

Step 2. Terminate the activation by addition of a *prewarmed reagent containing a chromogenic substrate*. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as a 50.0 per cent v/v solution of *acetic acid*, or a *1 M pH 3 citrate buffer solution*. Adjust the hydrolysis time to achieve a linear development of chromophore over time. Appropriate hydrolysis times are usually between 3 to 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Calculate the potency of the test preparation by the usual statistical methods (5.7).

2.8.7. Assay of Human Coagulation Factor IX

The principle of the assay is to measure the ability of a factor IX preparation to reduce the prolonged coagulation time of factor IX-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation under examination to that of a reference preparation, calibrated in International Units.

The International Unit is the factor IX activity of a stated amount of the International Standard, which consists of a freeze-dried concentrate of human coagulation factor IX. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human coagulation factor IX concentrate reference preparation is calibrated in International Units by comparison with the International Standard.

Reconstitute separately the preparation under examination and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (2.8.9) and neutralise the heparin, for example by addition of *protamine sulphate* (10 µg of protamine sulphate neutralises 1 IU of heparin). Predilute the preparation under examination and the reference preparation in factor IX-deficient plasma (for example *plasma substrate*) to produce solutions containing 0.5-2.0 IU per ml. Prepare at least 3 dilutions for each material, preferably in duplicate, using a suitable *buffer solution* (for example *imidazole buffer solution pH 7.3*) containing 1.0 per cent w/v of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or carry out the assay with incubation tubes maintained in a water-bath at 37°. Place in each tube 0.1 ml of factor IX-deficient plasma (for example *plasma substrate*) and 0.1 ml of one of the dilutions of the reference preparation or of the preparation under examination. Add to each tube 0.1 ml of a suitable *activated partial thromboplastin Time (APTT) reagent containing phospholipid and contact activator* and incubate the mixture for a recommended time at 37°. To each tube, add 0.1 ml of a 0.37 per cent w/v solution of *calcium chloride* previously heated to 37°. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the *calcium chloride* and the first indication of the formation of fibrin. The volumes given above may be adapted to the *APTT reagent* and apparatus used. Calculate the potency using the usual statistical methods (5.7).

2.8.8. Assay of Human Coagulation Factor X

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor X activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human coagulation factor X. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

Reagents

Russell's viper venom specific factor X activator (RVV). A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4° and use within 1 month.

Factor Xa chromogenic substrate. Specific chromogenic substrate for factor Xa such as: *N-á-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulphonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate.* Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 0.37 per cent w/v of *tris(hydroxymethyl)aminomethane*, 1.8 per cent w/v of *sodium chloride*, 0.21 per cent w/v of *imidazole*, 0.002 per cent w/v of *hexadimethrine bromide* and 0.1 per cent w/v of *bovine albumin* or *human albumin*. Adjust to pH 8.4 if necessary using *hydrochloric acid*.

Method

Test solution. Dilute the preparation under examination with *dilution buffer* to obtain a solution containing 0.18 IU of factor X per ml. Prepare at least 3 further dilutions with same solvent.

Reference solution. Dilute the reference preparation with *dilution buffer* to obtain a solution containing 0.18 IU of factor X per ml. Prepare at least 3 further dilutions with same solvent.

Warm all solutions to 37° in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37°, add 12.5 µl of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 µl of *RVV* and incubate for exactly 90 seconds. To each well add 150 µl of factor Xa chromogenic substrate, diluted 1 in 6 in *dilution buffer*.

Read the rate of change of absorbance (2.4.7) (at 405 nm continuously over a period of 3 minutes and obtain the mean rate of change of absorbance (ΔA per minutes). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 seconds, plot the absorbances against time on a linear graph and calculate ΔA per minute as the slope of the line. From the ΔA per minute values of each individual dilution of standard and test preparations, calculate the potency of the preparation under examination and check the validity of the assay by the usual statistical methods (5.7).

2.8.9. Assay of Heparin in Coagulation Factors

Heparin is assayed as a complex with antithrombin III (AT) via its inhibition of coagulation factor Xa (anti-Xa activity). An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralised by the heparin-AT complex and the residual factor Xa hydrolyses a specific chromogenic peptide substrate to release a chromophore. The quantity of chromophore is inversely proportional to the activity of the heparin.

Factor Xa chromogenic substrate. Specific chromogenic substrate for factor Xa such as: *N-benzoyl*-L-*isoleucyl*-L-*glutamyl-glycyl*-L-*arginine-4-nitroanilide hydrochloride*. Reconstitute according to the manufacturer's instructions.

Dilution buffer. 0.605 per cent w/v solution of *tris(hydroxymethyl)aminomethane*. Adjust to pH 8.4 if necessary using *hydrochloric acid*.

Test solution. Dilute the preparation under examination with *dilution buffer* to obtain a solution expected to contain 0.1 IU of heparin per ml.

Reference solution. Dilute the heparin reference preparation with *dilution buffer* to obtain a solution containing 0.1 IU of heparin per ml.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Warm all solutions to 37° in a water-bath shortly before the test.

Distribute in a series of wells, 20 µl of *normal human plasma* and 20 µl of *antithrombin III solution*. Add to the wells a series of volumes (20 µl, 60 µl, 100 µl and 140 µl) of the test solution or the reference solution and make up the volume in each well to 200 µl using *dilution buffer* (0.02-0.08 IU of heparin per ml in the final reaction mixture).

End-point method. Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution* and incubate at 37° for 30 seconds. Add 40 µl of a 1 mmol per litre solution of factor Xa chromogenic substrate and incubate at 37° for 3 minutes. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20.0 per cent *v/v* solution of *glacial acetic acid* and measure the absorbance (2.4.7) at 405 nm. Appropriate reaction times are usually between 3 to 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Kinetic method. Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution* and incubate at 37° for 30 seconds. Add 40 µl of a 2 mmol per litre solution of *factor Xa chromogenic substrate*, incubate at 37° and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (2.4.7), thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation by the usual statistical methods for a slope-ratio assay.

2.8.10. Determination of ABO Blood Group and Rh Group

A. Determination of ABO Blood Group of Blood Donors

ABO blood-grouping reagents

Source. ABO blood-grouping reagents are derived from the sera or defibrinated plasma of selected persons of appropriate ABO blood group who may have been deliberately immunised with either red corpuscles or group-specific substance of the appropriate blood group or groups, or derived from the sera of lower animals after immunisation or from cultures of mammalian lymphocytes. They are to be tested with negative results for the presence of hepatitis B surface antigen and antibodies to HIV-I and HIV-II by suitable sensitive methods approved by the appropriate authority.

Description. ABO blood-grouping reagents are of three types, namely Anti-A blood-grouping reagents, Anti-B blood-grouping reagent and Anti A, B (Group O) blood-grouping reagent. They may be issued as liquids or they may be prepared by reconstitution from the dried reagents.

Liquid Anti-A, or Anti-B, or Anti-A,B (Group O) reagents are clear or slightly opalescent yellowish or colourless fluids without turbidity. Anti-A reagent is artificially coloured blue or blue-green; anti-B reagent is artificially coloured yellow. They may contain a suitable antimicrobial preservative. Dried reagents are pale yellow powders or friable solids and are used after reconstitution.

Properties. Anti-A blood-grouping reagent agglutinates human red corpuscles containing A antigens, including sub-groups A_1, A_2, A_1B and A_2B, but rarely agglutinates those red corpuscles classified as Ax. It does not agglutinate human red corpuscles which do not contain A agglutinogens, i.e. red blood corpuscles of groups O and B. It also does not agglutinate group O or group B red corpuscles coated with IgG.

Anti-B blood-grouping reagent agglutinates human red corpuscles containing B antigens, i.e. red corpuscles of blood groups B and AB including sub-groups A_1B and A_2B. It does not agglutinate human red corpuscles, which do not contain B agglutinogens, i.e. red blood corpuscles of groups O, A_1 and A_2. It also does not agglutinate group O or group red Corpuscles coated with IgG.

Anti A,B (Group O) blood-grouping reagent agglutinates human red corpuscles containing A or B antigens, i.e. it agglutinates red corpuscles of blood groups A,B and AB including sub-groups A_1, A_2, A_x, A_1B, A_2B, and A_xb. It does not agglutinates human red corpuscles which do not contain A or B antigens, i.e. red corpuscles of blood group O. it also does not agglutinates group O red corpuscles coated with IgG

Anti-A, Ant-B and Anti-A, B (Group O) Blood grouping Reagents are shown not to contain antibodies to serum protein factors Gm or Km.

ABO blood-grouping reagents, reconstituted where necessary as stated on the label, comply with the following requirements.

Before use, everyday each vial of Blood grouping reagent must be checked for its appearance, specificity, avidity and potency with known fresh pooled cells.

Appearance. On visual inspection no hemolysis, precipitation, particle or gel formation is seen.

Specificity. ABO Blood-grouping reagent shows clear cut reactions with red cells having corresponding antigens and no reaction with negative control.

Avidity. A mixture of 1 volume of the reagent with 1 volume of a 5.0 to 10.0 per cent v/v suspension of human red corpuscles of each group or sub-group should not take more than twice the time that is taken for agglutination to appear first to the unaided eye when compared with the time taken when the appropriate International Standard of Blood-typing Serum or another suitable reference preparation of equivalent avidity in place of the reagent under examination.

Anti-A blood-grouping reagent — Use human red corpuscles of sub-groups A_1, A_2, A_1B and A_2B,. The appropriate International Standard is that for Anti-A blood-typing serum.

Anti-B blood-grouping reagent — Use human red corpuscles of sub-group B. The appropriate International Standard is that for Anti-B blood-typing serum.

Anti A,B (Group O) blood-grouping reagent — Use human red corpuscles of sub-groups A_1, A_2 and of group B. The appropriate International Standard is that for Anti-A, B blood-typing serum.

The following are the minimum requirements for the time taken by anti-A serum to show naked eye agglutination when mixed on a slide with an equal volume of a 5.0 to 10.0 per cent v/v suspension of A_1, A_2, and A_2B cells.

Serum	*Test corpuscles*	*Titre*
Anti-A	A_1	15 seconds
	A_2	30 seconds
	A_2B	45 seconds
Anti-B	B	15 seconds

Potency. The potency of an ABO blood-grouping reagent is determined by comparing its "saline agglutinin" antibody activity with that of the appropriate International Standard of Blood-typing serum or with that of another suitable reference preparation the potency of which has been determined in relation to the appropriate International Standard.

The determination is carried out by simultaneously titrating the reagent under examination and the reference preparation against suspension of human red corpuscles of the groups or sub-groups given below.

Anti-A, Anti-B, and Anti A, B (Group O) blood-grouping reagents should contain not less than 64 Units of the respective antibody per ml, i.e. for each type of red corpuscle against which it is titrated, the titre of the reagent under examination is not less than one quarter of that of the appropriate reconstituted International Standard irrespective of the actual titres obtained. (The International Standard for Blood-typing serum may be obtained from the WHO International Laboratory for Biological Standards, Central Laboratory, Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, The Netherlands).

The following are the minimum requirements for titre of anti-A serum with A_1, A_2, and A_2B corpuscles and of anti-B serum with B corpuscles.

Serum	*Test corpuscles*	*Titre*
Anti-A	A_1	256
	A_2	128
	A_2B	64
Anti-B	B	256

Storage. ABO blood-grouping reagents should be stored in sterile, light-resistant containers sealed so as to exclude microorganisms.

Liquid ABO blood-grouping reagents which do not contain an antimicrobial preservative should be kept frozen, preferably at a temperature below - 30° unless the preservative has been shown to be innocuous to the reagent in the frozen state.

Liquid ABO blood-grouping reagents containing an antimicrobial preservative should be kept at a temperature between 2° and 8°.

Dried ABO blood-grouping reagents should be kept at a temperature not higher than 20°.

Labelling. The label states (1) the name "Anti-A blood-grouping reagent" printed on blue paper, "Anti-B blood-grouping reagent" printed on yellow paper, or "Anti-A,B (Group O) blood-grouping reagent" as appropriate; (2) the number of Units of the relevant antibody or antibodies per ml; (3) the batch number; (4) the quantity in the container; (5) the

date after which the preparation may not expected to retain its activity; (6) the storage conditions; (7) for a liquid reagent containing an antimicrobial preservative, the name and concentration of the preservative used, and that the reagent should not be frozen unless the preservative has been shown to be innocuous to the reagents in the frozen state; (8) for dried reagents the nature and volume of the liquid to be used for reconstitution.

Method

The blood is examined both for agglutinogens of the red corpuscles and for agglutinins in the serum. A few ml of fresh blood, without anticoagulant, is allowed to clot in a narrow test-tube, and the serum removed. The clot or a small part of it is stirred with *saline solution*, the resulting suspension centrifuged and the corpuscles resuspended in *saline solution* to make a 2.0 to 3.0 per cent v/v suspension of packed corpuscles.

Test for agglutinogens. The test is conveniently made in lipless tubes (50 mm x 6 mm), each containing 0.04 ml volumes of diagnostic serum, of the red corpuscles suspension and of *saline solution* (to avoid nonspecific agglutination) delivered from calibrated Pasteur pipettes. One tube contains anti-A serum and a second contains anti-B serum.

It is advisable to control the specificity of anti-A serum and anti-B serum by testing them with red corpuscles of known groups A, B and O.

The tubes are shaken and left at room temperature for 2 hours, covered with cylindrical glass caps to prevent evaporation and to protect the contents. The caps are then removed and each tube flicked with a finger to disperse the deposit of corpuscles. The contents of tubes in which no obvious agglutination has occurred are examined microscopically at a magnification of x50 to x100, spread evenly on a slide with the stem of a Pasteur pipette. The reaction is scored as follows.

++++ = Complete agglutination into one or two large clumps.

+++ = Numerous clumps clearly visible to the naked eye.

++ = Granularity just visible to the naked eye; very big clumps and unagglutinated corpuscles seen under the microscope.

+ = Not quite such big clumps; numerous unagglutinated corpuscles.

(+) = Clumps of 8 to 12 corpuscles.

w = A definite but weak reaction in which there is a uniform distribution of very small clumps of 4 to 6 corpuscles.

? = Uneven distribution of corpuscles with no definite clumps.

\- = All corpuscles separate and evenly distributed.

In test of red corpuscles a reaction less than + is unlikely to be due to A or B agglutinogens, and the corpuscles should be tested.

Test for agglutinins. The serum of the blood under examination is similarly tested against group A and B red corpuscles, 0.04 ml of the suspension and 0.04 ml of the undiluted serum being used. In this test, small reactions are frequent, and in about 50.0 per cent of infants under 1 year old, either one or both of the agglutinins to be expected on the basis of agglutinogens of the red corpuscles, are not found. In all bloods, a *"w"* reaction is considered to be true, a "?" requires a retest. When the expected agglutinins are not disclosed after retest, the diagnosis is based on the agglutinogens of the red corpuscles.

Slide or tile method. Blood obtained from the finger-prick is diluted with *saline solution* so as to give a suspension containing approximately 5.0 per cent v/v red corpuscles. Oxalated blood is also suitable for this purpose.

Take two plain glass slides marked A and B, place one drop of red cell suspension on each of them, add one drop of anti-A serum on the slide marked B. Mix with a glass rod and gently rock the slides to and fro. The agglutinate appears almost at once with the appropriate serum.

Negative readings should not be taken until the preparation has stood at least for 10 minutes. The reaction is visible to the naked eye but a hand lens or a low power microscope may be employed in doubtful cases. When a large number of blood samples have to be tested for blood groups, a long porcelain or opal glass tile may be employed in place of glass slides.

Weak reactions with anti-A serum in group AB. Care must be taken in making the diagnosis of group B because the reaction of anti-A serum with the A agglutinogens is often weak when the B agglutinogen is also present on the corpuscles; group AB corpuscles may therefore be falsely diagnosed as group B corpuscles. This is particularly true of the sub–group A, B in which the reactions of the A agglutinins are always weak. The examination of the serum for agglutinins does not necessarily serve as a check since the serum of a number of A, B persons contains the agglutinin anti-A, which reacts with A_1 but not with A_2 corpuscles. Since A_1 corpuscles are used to check the serum, agglutination may occur and the diagnosis of group B corpuscles be apparently confirmed. The mistake is, however, unlikely to arise if an anti-A serum is used which has been tested for its capacity to react with known AB corpuscles. As an additional check the serum from every blood that has been shown by determination of its agglutinogen and agglutininin content to belong to group B is tested against known A_2

corpuscles. If there is a reaction it is clear that the serum must contain the agglutinin alpha, which reacts with A_2 as well as with A_1 corpuscles. The serum, therefore, cannot have come from an A_1B or an A_2B person and must have come from a group B person. The diagnosis that corpuseles belong to group B should be made unless the serum reacts with A_2 corpuscles.

ABO group of blood is determined from the antigens on the red corpuscles and the antibodies in the serum or plasma. Collect a few ml of blood without anticoagulant in a test-tube and allow to clot. Remove the serum and prepare a 2.0 to 3.0 per cent suspension of red corpuscles in *saline solution*.

Test for antigens. Mix 1 volume of the red corpuscle suspension with 1 volume of *anti-A blood-grouping reagent*, *anti-B blood-grouping reagent* and *anti-A,B (group O) blood-grouping reagent*, the specificities of which have been demonstrated by testing with known red corpuscles of group A,B and O. Allow the mixture in the test-tube to stand at room temperature for 1 to 2 hours and then tap them gently to disperse the deposit of corpuscles. Examine the contents of the tubes microscopically for agglutination.

Test for antibodies. Mix separate portions of the serum or plasma with suspension of human red corpuscles of group A (sub-groups A_1 and A_2), group B and group O and determine the antibodies present by examination of the resulting pattern of deposit of corpuscles. As antibodies are incompletely developed in children under one year of age, the blood group of the child is based solely on the antigens present on red corpuscles.

B. Determination of Rh Group of Donors

Rh Blood-Grouping Reagents

Source. Rh blood-grouping reagents are derived from the sera or defibrinated plasma of one or more persons immunised by Rh system or from cultures of mammalian lymphocytes. The material to be used must be tested with negative results for the presence of hepatitis B surface antigens and anti-bodies to HIV I and HIV-II by suitable sensitive methods approved by the appropriate authority.

Description. Rh blood-grouping reagents may be issued as liquids or they may be prepared by reconstitution from the dried reagents. Liquid reagents are clear or faintly opalescent, yellowish or colourless fluids without turbidity. They may contain a suitable antimicrobial preservative. Dried reagents are pale yellow powders or friable solids.

Rh blood-grouping reagents are of four types, namely IgM Anti-D blood-grouping reagent, IgG Anti-D blood grouping reagent, Anti-C blood-grouping reagent and Anti-E blood-grouping reagent.

Properties. Blended IgG and IgM monoclonal reagents are preferably used for Rh blood-grouping in blood banks. Anti-D, Anti-C and Anti-E blood-grouping reagent should be shown not to agglutinate or to coat any of a comprehensive panel of corpuscles that do not contain the respective antigens.

Rh blood-grouping reagents, reconstituted where necessary as stated on the label, comply with the following requirements.

Potency. *IgM anti-D blood-grouping reagent* — It contains anti-D as a "saline agglutinin" in such quantities that it gives a positive reaction at a dilution of 1 in 32 against corpuscles known to contain the D antigens.

IgG anti-D blood-grouping reagent — The potency of IgG anti-D blood-grouping reagent is determined by comparing its "albumin agglutinin" antibody activity with that of the appropriate International Standard of Blood-typing serum or with that of another suitable reference preparation the potency of which has been determined in relation to the appropriate International Standard.

The determination is carried out by simultaneously titrating the reagent under examination and the reference preparation against suspension of human red corpuscles containing the D antigens.

IgG anti-D blood-grouping reagent contains not less than 32 Units of anti-D antibody per ml, i.e. the titre of the reagent under examination is not less than one-half of that of the appropriate reconstituted International Standard irrespective of the titre obtained.

Anti-C blood-grouping reagent—It contains anti-C antibody in such quantities that it gives a positive reaction at a dilution of I in 8 with red corpuscles known to contain the C antigens.

Anti-E blood-grouping reagent — It contains anti-E antibody in such quantities that it gives a positive reaction at a dilution of 1 in 8 with red corpuscles known to contain the E antigens.

Storage. Rh blood-grouping reagents should be kept in sterile containers sealed so as to exclude micro-organisms.

Liquid Rh blood-grouping reagent which do not contain antimicrobial preservative should be kept frozen, preferably at a temperature below -30°.

Liquid Rh blood-grouping reagents containing an antimicrobial preservative should be kept at a temperature between 2° and 8° ; they should not be frozen unless the antimicrobial preservative has been shown to be innocuous to the reagent in the Frozen state.

Dried Rh blood-grouping reagents should be kept at a temperature not higher than 20°.

Labelling. The label states (1) the name "IgM Anti-D blood-grouping reagent", "IgG Anti-D blood-grouping reagent",

"Anti-C blood-grouping reagent" or "Anti-E blood-grouping reagent" as appropriate; (2) the agglutination titre; (3) the batch number; (4) the date after which the preparation may not be expected to retain its activity; (5) the storage conditions; (6) for a liquid reagent containing an antimicrobial preservative, the name and concentration of the preservative used and that the reagent must not be frozen unless the preservative has been shown to be innocuous to the reagent in the frozen state; (7) for dried reagents the nature and volume of the liquid to be used for reconstitution.

Method. Since the haemagglutinins of the Rh system are present in human blood only in pathological conditions, it is necessary to depend solely on the reaction of the red corpuscles in order to determine the Rh group of a specimen of blood. Red corpuscles are obtained either by bleeding into a 3.0 per cent w/v solution of *sodium citrate* or by re-suspending in *saline solution* to give a suspension containing approximately 2.0 per cent v/v suspension of packed corpuscles.

In order to select Rh negative Blood, the untested donor blood is first tested with anti-D Rh serum and, in order to conserve these very rare sera, only those bloods which are unagglutinated are further tested with anti-C Rh serum and anti-E Rh serum.

For economical routine work, 0.01 ml volumes each of serum and corpuscles suspension are used, though for inexperienced workers, 0.04 ml volumes are preferable. With a Pasteur pipette, graduated at 0.01 ml intervals, 0.01 ml of anti-D Rh serum is deposited at the bottom of lipless test-tubes (50 mm x 6 mm); 0.01 ml of red corpuscles suspension is then deposited on the wall of the tube about 4 mm above the serum and the two are mixed and incubated for 2 hours at 37°. Control test of the serum are made with D-positive and D-negative corpuscles. For reading, the deposited corpuscles are gently taken up with a Pasteur pipette, spread evenly on a slide with the stem of the pipette, and examined microscopically at a magnification of x50 to x100. D-positive corpuscles form large clumps whereas D-negative corpuscles are unagglutinated. The tests with anti-C Rh serum and anti-E Rh serum are made similarly but, since the agglutination is usually weak, the deposited corpuscles must be transferred to the slide with minimum manipulation.

It is advisable to control the specificity of each anti-Rh serum used by testing it with red corpuscles of Rh groups that should react positively and of Rh groups that should react negatively with each serum.

2.8.11. Determination of Haemoglobin by Photometry

The determination of haemoglobin in blood is done by the measurement of colour of cyanmethaemoglobin obtained by conversion of haemoglobin to cyanmethaenoglobin by means of a special reagent.

Special Reagent

Ferricyanide-cyanide reagent. Dissolve 0.2 g of *potassium ferricyanide*, 50 mg of *potassium cyanide* and 0.14 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml. Check the pH and adjust, if necessary, to 7.2 ± 0.2. Store in a polyethylene bottle in the dark at a temperature below 20°.

NOTE — The solution should not be frozen.

Method

Pipette 0.02 ml of the substance under examination into a stoppered tube, add 4.0 ml of *ferricyanide cyanide reagent* and mix well. Allow to stand for 10 minutes and measure the absorbance (2.4.7) of the resulting solution at about 540 nm, using as blank the *ferricyanide-cyanide reagent*. Calculate the content of haemoglobin from the absorbance obtained by carrying out the determination simultaneously using a suitable volume of *cyanmethaemoglobin RS* and from the declared content of haemoglobin in *cyanmethaemoglobin RS*.

2.8.12. Assay of Human Anti-thrombin III

The anti-thrombin III content of the preparation under examination is determined by comparing its ability to inactivate thrombin in presence of an excess of heparin with the same ability of a reference preparation of human anti-thrombin III concentrate calibrated in International Units. Varying quantities of the preparation under examination are mixed with a given quantity of thrombin and the remaining thrombin activity is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of International Standard for human anti- thrombin III concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Method

Prepare 2 independent series of 3 or 4 dilutions in the range of 1/75 to 1/200 from 1 IU per ml, for both the preparation under examination and the reference preparation, using *tris-EDTA BSA buffer solution pH 8.4* containing 15 IU of heparin per millilitre.

Warm 200 µl of each dilution at 37° for 1-2 minutes. Add to each dilution 200 µl of a solution of bovine thrombin containing 2 IU per ml in *tris-EDTA BSA buffer solution pH 8.4*. Mix and maintain at 37° for exactly 1 minute. Add 500 µl of a suitable *chromogenic substrate* (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide reconstituted in *water* to

give a solution containing 4mmol per litre and further diluted to a concentration suitable for the assay using *tris-EDTA BSA buffer solution pH 8.4* without albumin). Immediately start measurement of the change in absorbance at 405 nm, continuing the measurement for at least 30 seconds. Calculate the rate of change of absorbance (DA per minute). (Alternatively, an end-point assay may be used by stopping the reaction with *acetic acid* and measuring the absorbance at 405 nm).

The rate of change of absorbance (DA per minute) is inversely proportional to anti-thrombin III activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (5.7).

2.8.13. Anti-A and Anti-B Haemagglutinins (Indirect Method)

Prepare in duplicate serial dilutions of the preparation under examination in a 0.9 per cent w/v solution of *sodium chloride*. To each dilution of one series, add an equal volume of a 5 per cent v/v suspension of group A_1 red blood cells previously washed three times with the *sodium chloride solution*. To each dilution of the other series add an equal volume of a 5 per cent v/v suspension of group B red blood cells previously washed three times with the *sodium chloride solution*. Incubate the suspensions at 37° for 30 minutes then wash the cells three times with the *sodium chloride solution*. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope.

2.8.14. Assay of Human Coagulation Factor XI

The principle of the assay is to measure the ability of a factor XI preparation to reduce the prolonged coagulation time of factor XI- deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipids and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation under examination to that of a reference preparation consisting of human normal plasma.

1 unit of factor XI is equal to the activity of 1 ml of human normal plasma. Human normal plasma is prepared by pooling plasma units from not fewer than 30 donors and stored at −30° or lower.

Reconstitute separately the preparation under examination and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (2.8.9) and neutralize the heparin, e.g. by addition of *protamine sulphate* (10 µg of protamine sulphate neutralizes 1 IU of heparin). Pre-dilute the preparation under examination and the reference preparation in factor XI- deficient plasma (eg. *plasma substrate*) to produce solutions containing 0.5-0.2 units per ml. Prepare at least 3 appropriate dilutions for each material, preferably in duplicate, using a suitable buffer solution (eg. *imidazole buffer solution pH 7.3*) containing 1 per cent w/v solution of bovine or human albumin. Use these dilutions immediately.

2.8.15. Assay of Human Protein C

A. Chromogenic assay

Human protein C is a vitamin K-dependant plasma protein that, upon activation to activated protein C (APC), can inhibit blood coagulation through proteolytic cleavage of factors Va and VIIIa. Human protein C in the preparation is activated by a specific activator from snake venom; in the 2nd step, APC cleaves a specific chromogenic substrate to forma chromophore that can be quantified spectrophotometrically.

Step 1

$$\text{Human protein C} \xrightarrow{\text{human protein activator}} \text{APC}$$

Step 2

$$\text{Chromogenic substrate} \xrightarrow{\text{APC}} \text{Peptide} + \text{Chromophore}$$

The potency of human protein C is estimated by comparing the ability of the preparation under examination to cleave a chromogenic substrate with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre plate end-point method.

Reagents

Dilution buffer pH 8.4. Dissolve 6.055 g of *tris(hydroxymethyl)aminomethane* and 16.84 g of *caesium chloride* in *water* and adjust the pH (2.2.3) if necessary. Dilute to 1000.0 ml with *water*.

Human protein C activator. Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions. Dilute to 0.25 U per ml with *water* before use in the assay.

Activated protein C chromogenic substrate. Specific chromogenic substrate for APC, for example L-pyroglutamyl-L-prolyl-l-arginine-paranitroaniline hydrochloride (pyroGlu-Pro-Arg-pNA.HCl). Reconstitute with *water* to give a concentration of 4.5 mmol per litre. Further dilute to 1.1 mmol per litre with dilution buffer pH 8.4 before use in the assay.

Method

Reconstitute or thaw the preparation under examination according to the manufacturer's instructions. Dilute with *water* to produce at least 3 separate dilutions for each preparation in the range 0.05-0.2 IU per ml, preferably in duplicate.

Step 1. Mix 0.025 ml of each dilution with 0.05 ml of the *human protein C activator*, both previously heated to 37°, and leave at 37° for exactly 10 minutes. For each dilution, prepare a blank in the same manner, using *water* instead of the *human protein C activator*.

Step 2. Add 0.150 ml of diluted chromogenic substrate, previously heated to 37°, to each mixture and leave at 37° for exactly 10 minutes. The incubation time must be adjusted, if necessary, to ensure a linear development of chromophore with time. Terminate the reaction by adding 0.05 ml of a 50 per cent v/v solution of *glacial acetic acid*.

Cleavage of the chromogenic substrate by APC causes release of the chromophore pNA, in proportion to the concentration of human protein C in the preparation. Measure the optical density at a wavelength of 405 nm. Subtract the optical density of the blank from the optical density of the test sample. Check the validity of the assay and calculate the potency of the preparation under examination using the usual statistical methods (5.7).

B. Clotting assay

Human protein C activity is estimated following cleavage to APC by a specific activator extracted from the venom of the viper *Agkistron contortrix*. The resulting APC inactivates factors Va and VIIIa, and thus prolongs the APTT (Activated Partial Thromboplastin Time) of a system in which all the coagulation factors are present, constant and in excess, except for human protein C, which is derived from the preparation being tested. Prolongation of the clotting time is proportional to the concentration of human protein C in the preparation.

The potency of human protein C is estimated by comparing the ability of the preparation under examination to prolong the clotting time with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

Reagents

Dilution buffer pH 7.4. Isotonic non-chelating buffer.

Human protein C-deficient plasma. Citrated human plasma with no measurable human protein C content. Reconstitute and store according to the manufacturer's instructions.

Human protein C activator. Protein isolated from the venom of the viper *Agkistrodon contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions.

Coagulation activator. A suitable APTT reagent containing phospholipids and a contact activator may be used. It may be combined with the *human protein C activator*.

Method

Reconstitute or thaw the preparation under examination according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.01 to 0.15 IU per ml, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein C-deficient plasma and 1 volume of the human protein C activator (combined with the APTT reagent where appropriate), all previously heated to 37°. Add 1 volume of *calcium chloride* previously heated to 37° and record the clotting time.

The clotting time is proportional to the concentration of human protein C in each dilution. Check the validity of the assay and calculate the potency of the preparation under examination using the usual statistical methods (5.7).

2.8.16. Assay of Human Protein S

Human protein S is a vitamin K-dependent plasma protein that acts as a cofactor for the anticoagulant functions of activated protein C (APC). Human protein S activity may be determined by the clotting assay described below, which is sensitive to the ability of human protein S to accelerate the inactivation of factor Va by APC. In practice, the assay involves the addition of human protein S to a reagent mixture containing APC, factor Va and human protein S-deficient plasma. Prolongation of the clotting time is proportional to the concentration of human protein S in the preparation. Methods in which APC is added directly as a reagent are preferred to those in which APC is generated during the assay by the addition of a specific *human protein C activator* purified from

snake venom. Activation of coagulation is initiated by the addition of an activating reagent such as thromboplastin or activated factor X, together with phospholipids and calcium chloride. During the assay, factor Va is generated from factor V in the human protein S-deficient plasma following the activation of coagulation. The assay procedure must ensure that human protein S is the only limiting factor.

The potency of human protein S is estimated by comparing the ability of the preparation under examination to prolong the clotting time with the same ability of a reference standard of human protein S calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein S. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

Reagents

Dilution buffer pH 7.4. Isotonic non-chelating buffer prepared as follows: dissolve 6.08g of *tris(hydroxymethyl) aminomethane* and 8.77g of *sodium chloride* in *water* and adjust the pH (2.2.3) if necessary; add 10 g of *bovine albumin* or *human albumin* and dilute to 1000.0 ml with *water*.

Human protein S-deficient plasma. Citrated human plasma with no measurable human protein S content and, preferably, also free of C4b-binding protein.

Coagulation activator. This reagent is used to initiate coagulation in the human protein S-deficient plasma, and thereby also provides a source of activated factor V. The activator may consist of tissue factor, activated factor X, or an agent capable of directly activating factor X that may be purified from the venom of *Russell's viper (Vipera russelli)*. The reagent also contains APC, phospholipids and *calcium chloride* or alternatively, *calcium chloride* may be added separately after a timed activation period.

Method

Reconstitute or thaw the preparation under examination according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.02 to 0.1 IU per ml, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein S-deficient plasma, both previously heated to 37°. Add 2 volumes of the coagulation activator, previously heated to 37°, and record the clotting time.

Alternative procedures may use a coagulation activator without *calcium chloride*, and require a precisely timed activation period before the addition of *calcium chloride* and the measurement of clotting time.

The clotting time is proportional to the concentration of human protein S in each dilution. Check the validity of the assay and calculate the potency of the preparation under examination using the usual statistical methods (5.7).

2.8.17. Assay of Human Plasmin Inhibitor (α_2-Antiplasmin)

Human plasmin inhibitor, also called human a_2-antiplasmin, is a plasma protein that inhibits the plasmin (a serine protease) pathway of fibrinolysis by rapidly forming a complex with free plasmin. Furthermore, upon blood coagulation, human plasmin inhibitor is cross-linked to fibrin strands by factor XIII, and interferes with binding of the proenzyme plasminogen to fibrin.

The potency of human plasmin inhibitor is estimated by comparing the ability of the preparation under examination to inhibit the cleavage of a specific chromogenic substrate by plasmin with the same ability of a reference standard of human plasmin inhibitor. Plasmin cleavage of the chromogenic substrate yields a chromophore that can be quantified spectrophotometrically.

The individual reagents for the assay may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

Reagents

Dilution buffer pH 7.5. A suitable buffer is used. Adjust the pH, if necessary.

Plasmin. A preparation of human plasmin that does not contain significant amounts of other proteases is preferably used. Reconstitute and store according to the manufacturer's instructions.

Plasmin chromogenic substrate. A suitable specific chromogenic substrate for plasmin is used: H-D-cyclohexylalanyl-norvalyl-lysyl-*p*-nitroaniline hydrochloride (H-D- CHA-Nva-Lys-*p*NA.HCl) or L-pyroglutamyl-L-phenylalanyl-L-lysine-*p*-nitroaniline hydrochloride (Glp-Phe-Lys-*p*NA.HCl). Reconstitute in *water* to give a suitable concentration according to the manufacturer's instructions.

Method

Varying quantities of the preparation under examination are mixed with a given quantity of plasmin and the remaining

plasmin activity is determined using a suitable chromogenic substrate.

Reconstitute or thaw the preparation under examination according to the manufacturer's instructions. Dilute with dilution buffer pH 7.5 and prepare at least 2 independent series of 3 or 4 dilutions for both the preparation under examination and the reference standard.

Mix 0.02 ml of each dilution with 0.02 ml of dilution buffer pH 7.5 and warm to 37°. Add 0.04 ml of a plasmin solution (test concentration in the range of 0.2 nkat per ml to 1.6 nkat per ml) previously heated to 37° and leave at 37° for 1 minute. Add 0.02 ml of the chromogenic substrate solution, previously heated to 37°, to each mixture. Immediately start measurement of the change in absorbance at 405 nm (2.4.7) using a microtitre plate reader. Calculate the rate of change of absorbance (DA per minute). Alternatively, an end-point assay might be used by stopping the reaction with *acetic acid* and measuring the absorbance at 405 nm.

In both cases the duration of the cleavage of the chromogenic substrate should be chosen to produce a linear increase in absorbance at 405 nm, before substrate depletion becomes significant. If the assay is performed in test tubes or cuvettes using a spectrophotometric method, the volumes of reagent solutions are changed proportionally.

Substract the optical density of the blank (prepared with dilution buffer pH 7.5) from the optical density of the preparation under examination. Check the validity of the assay and calculate the potency of the preparation under examination by the usual statistical methods (5.7).

3. REFERENCE DATA

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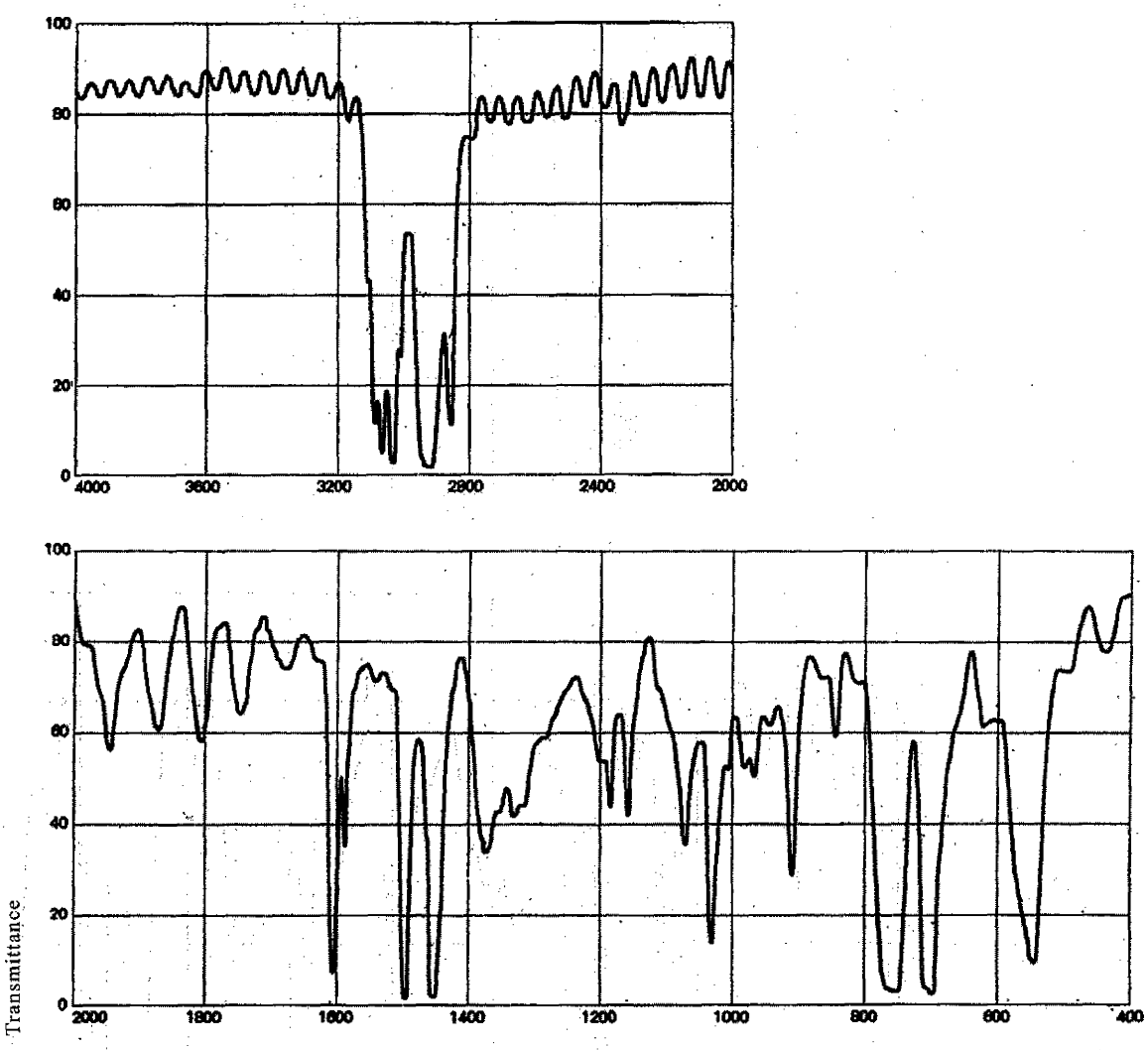
3.1. Infra-red Reference Spectra

The spectra were recorded either on a Dispersive Infra-Red Spectrophotometer (Dispersive IR) or a Fourier Transform Infra-red Spectrophotometer (FTIR). Comparisons can be made between a spectrum obtained with a dispersive instrument and a reference spectrum produced on a FT instrument and vice versa. Where a reference spectrum is not included in this section, the spectrum obtained from the substance under examination has to be compared with the spectrum obtained from the corresponding reference substance.

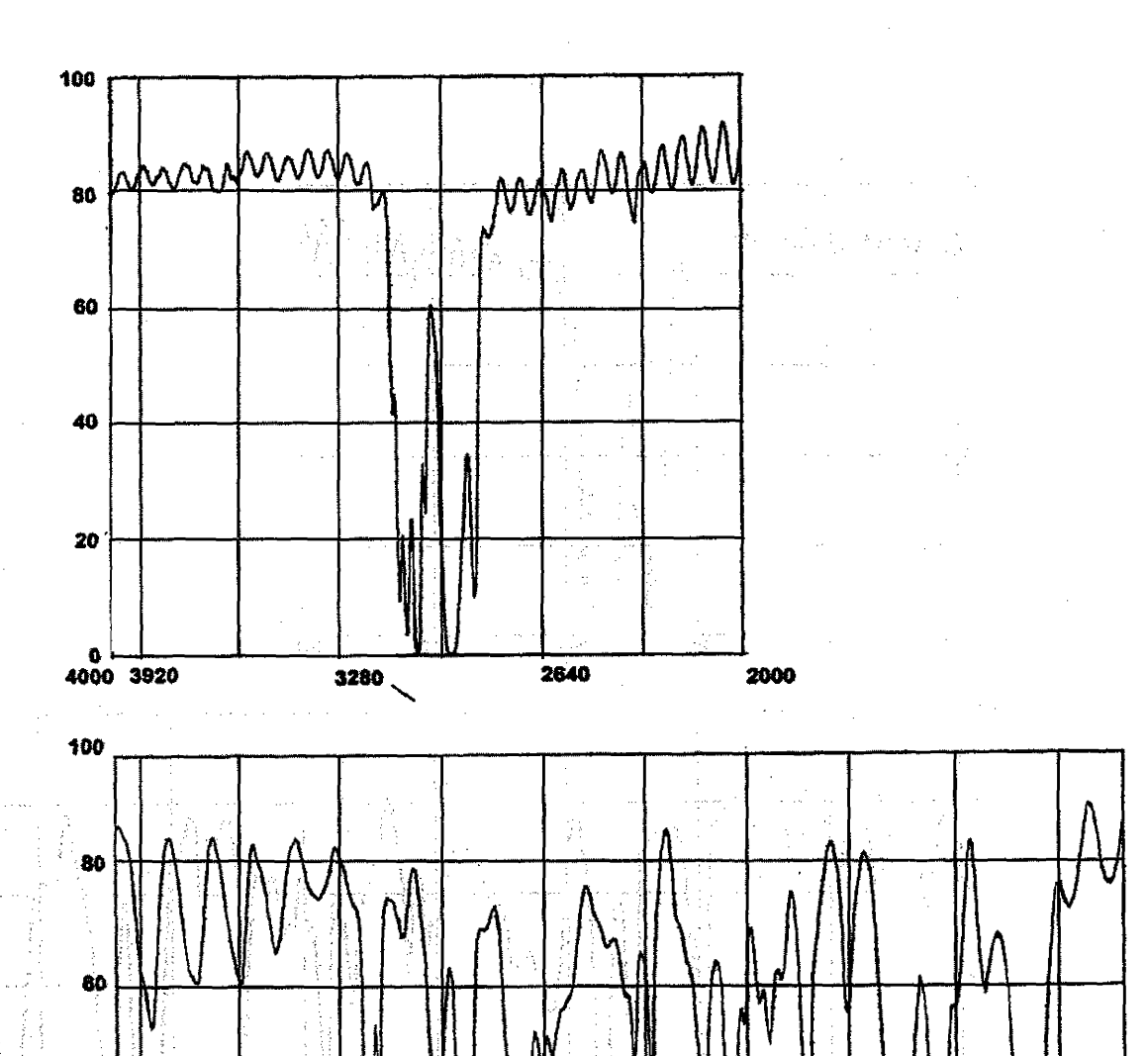
Solid state spectra were recorded against air with liquid paraffin mulls placed between potassium bromide plates or with pressed discs, 13 mm in diameter, using potassium bromide or potassium chloride. Solution spectra were recorded against a solvent reference using cells with potassium bromide windows. The instrument and the Phase: in which the spectra were recorded are indicated on top of the individual reference spectrum.

For solution spectra the absorption bands due to the substance under examination that coincide with strong solvent absorption will not be recorded, as stated in (2.4.6). Solvent 'cut-offs' in the reference spectra in such cases are recorded as horizontal straight lines.

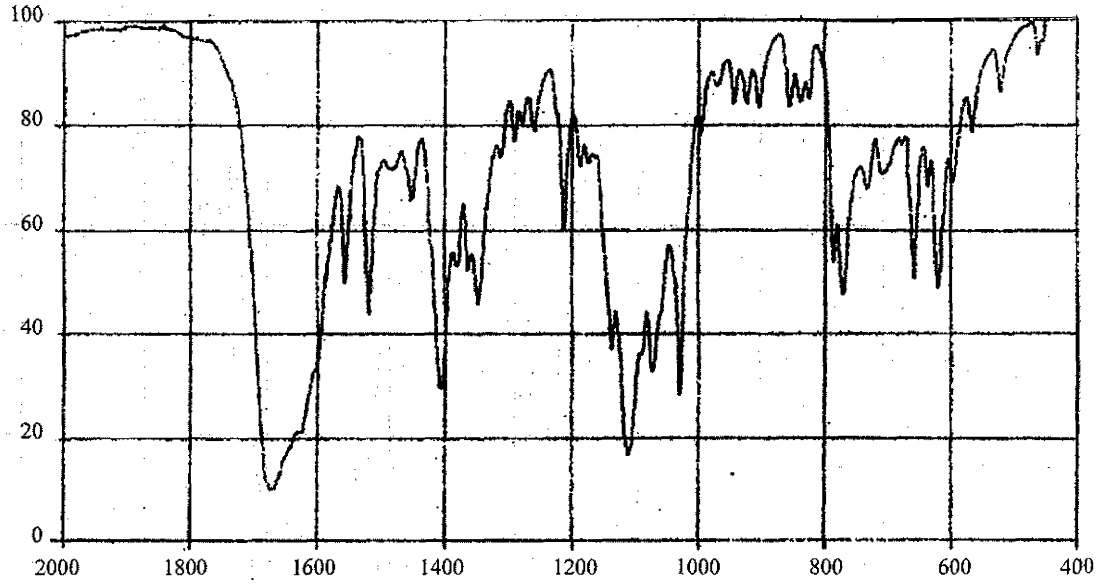
Polystyrene *Dispersive IR* Phase: Thin film Thickness 0.05 mm



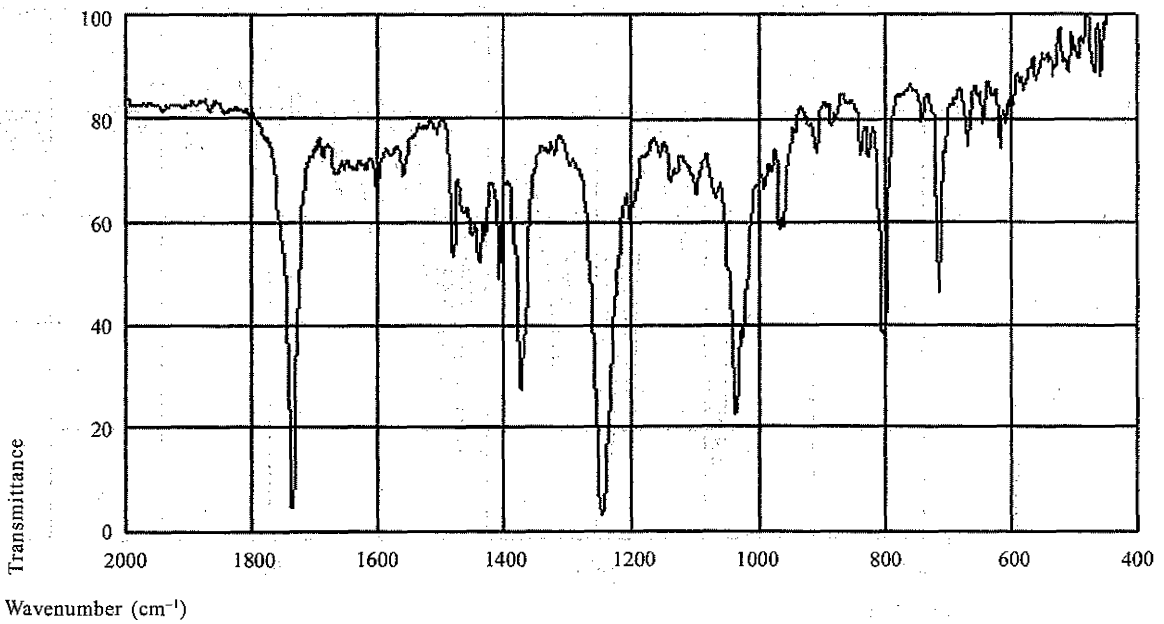
Polystyrene *FTIR* Phase: Thin film Thickness 0.05 mm



Abacavir Sulphate *FTIR* Phase: KBr disc



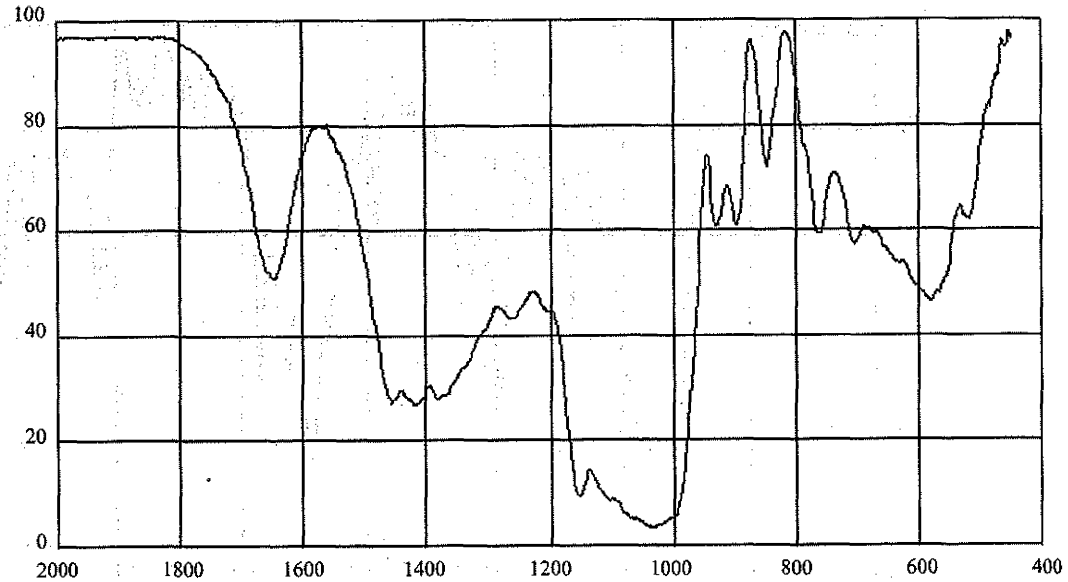
Abiraterone Acetate *FTIR* Phase: KBr disc



Acarbose

FTIR

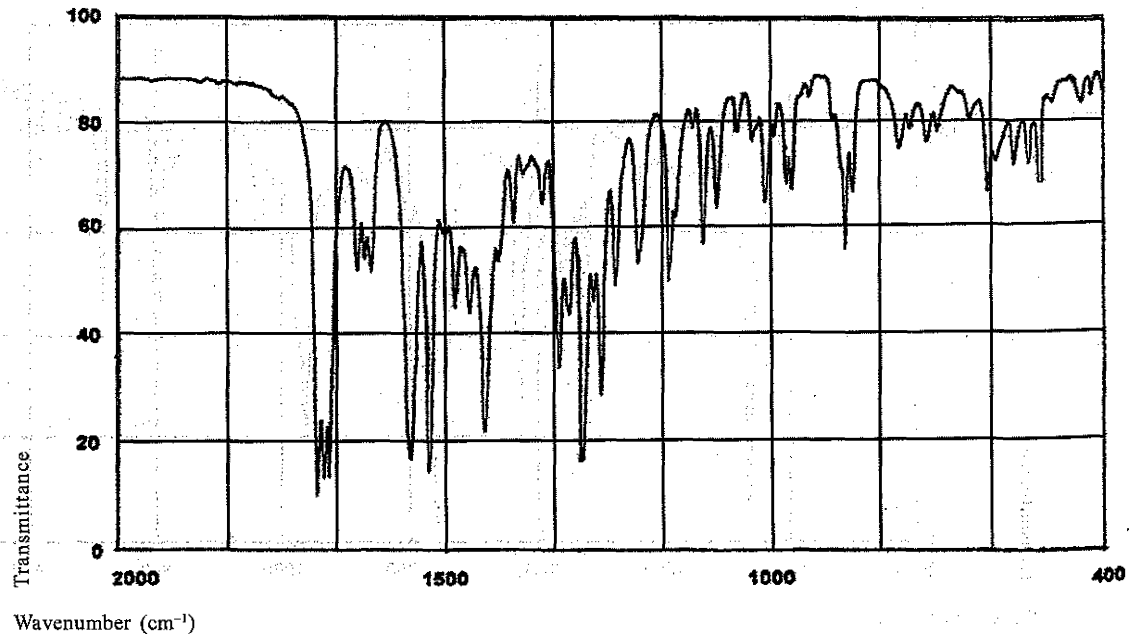
Phase: KBr disc



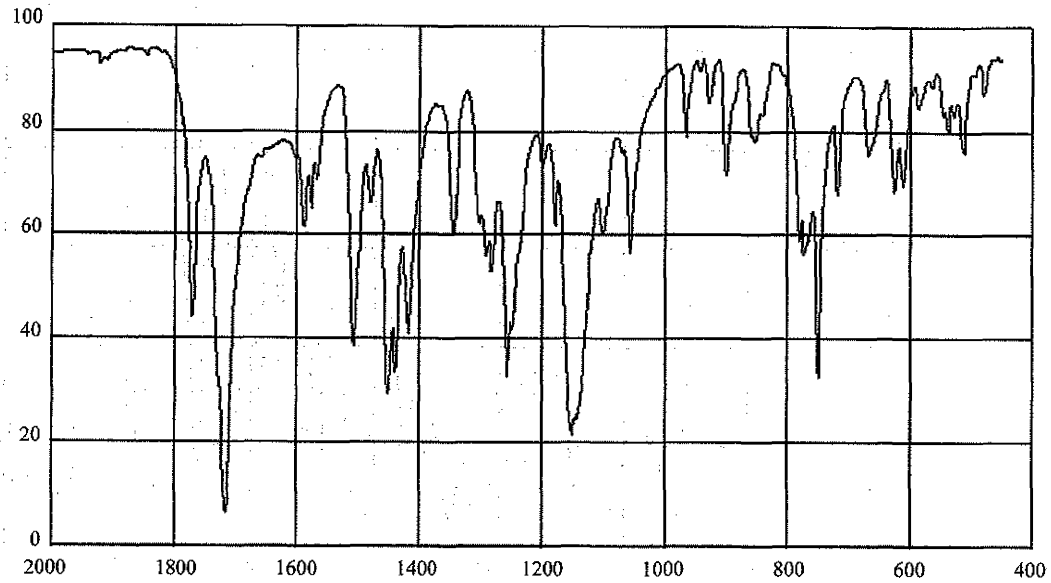
Acebutolol Hydrochloride

FTIR

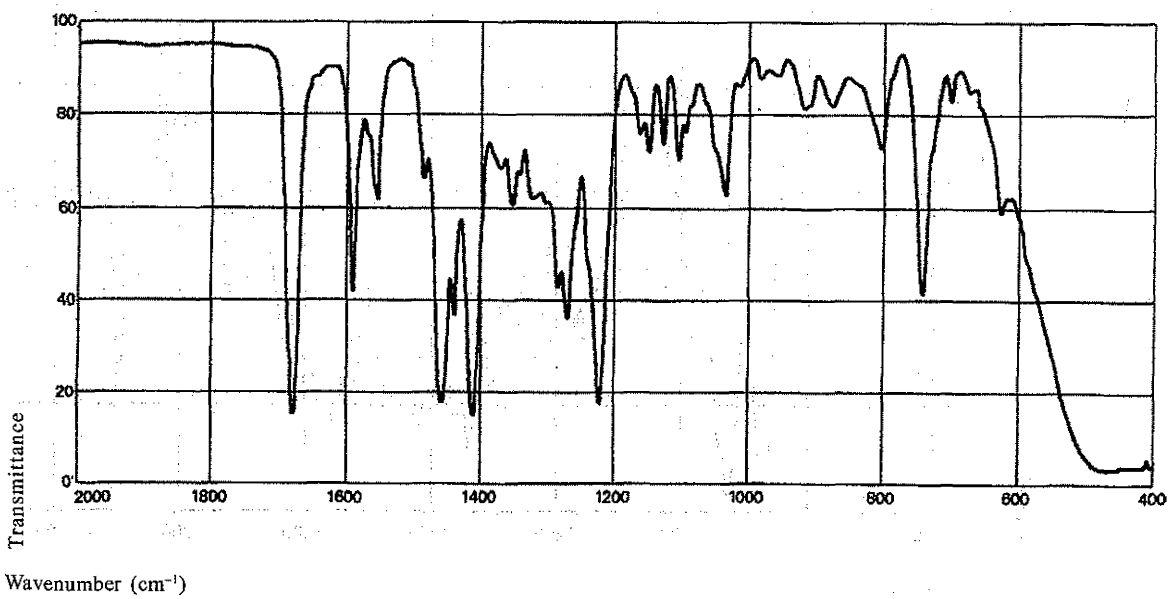
Phase: KBr disc



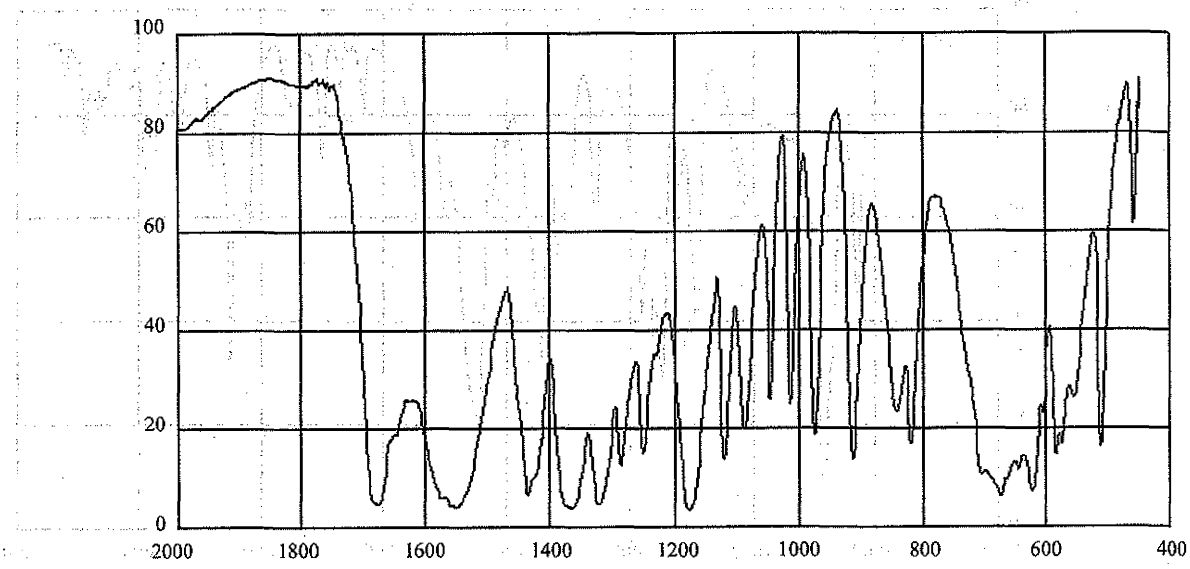
Aceclofenac *FTIR* Phase: KBr disc



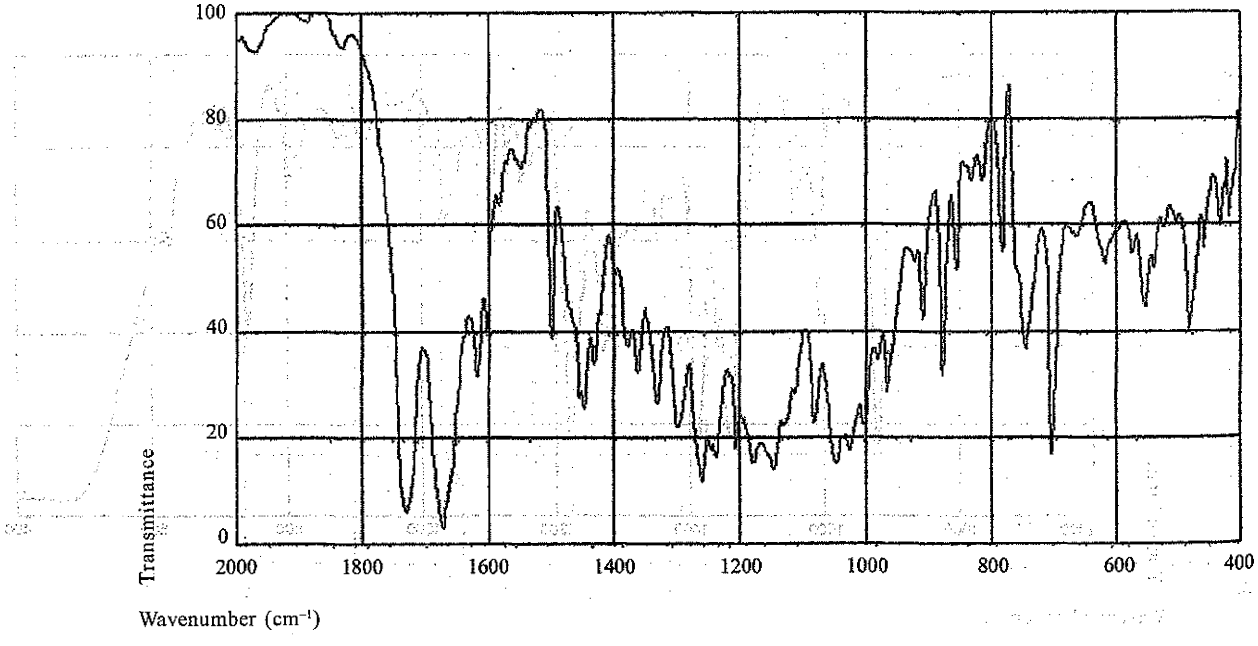
Acepromazine *Dispersive IR* Phase: Thin film



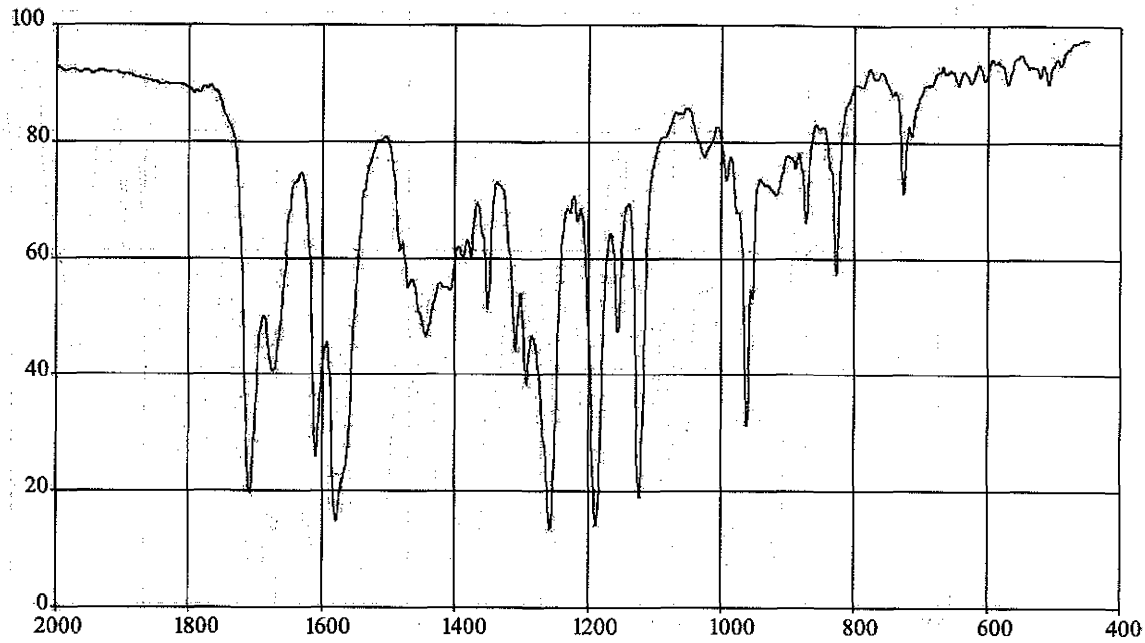
Acetazolamide *FTIR* Phase: KBr disc



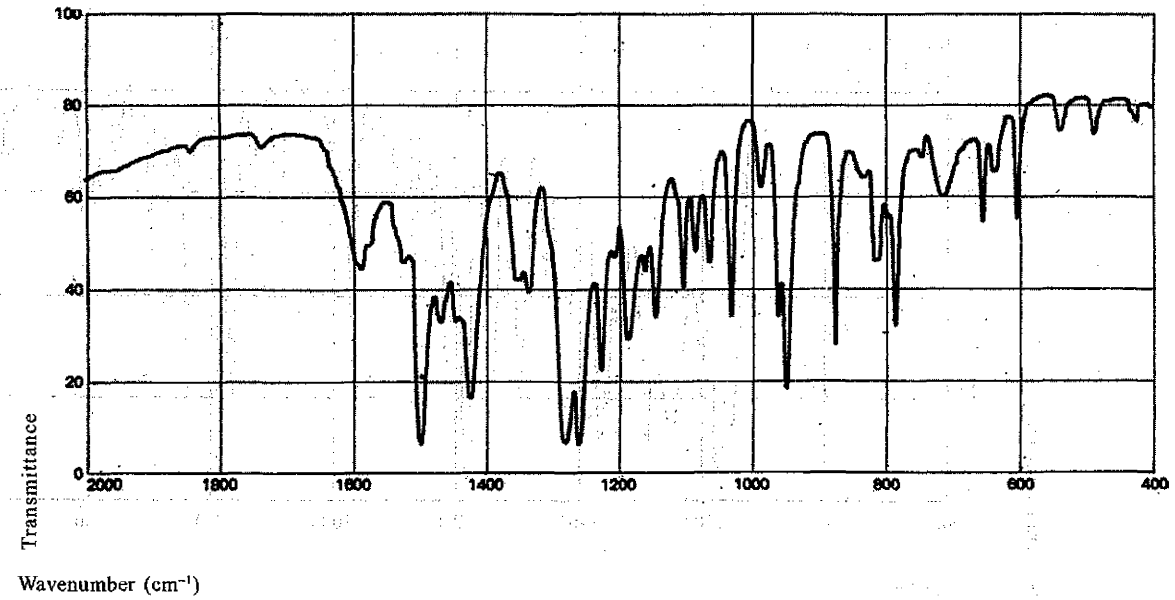
Aciclovir *FTIR* Phase: KBr disc



Acitretin *FTIR* Phase: KBr disc



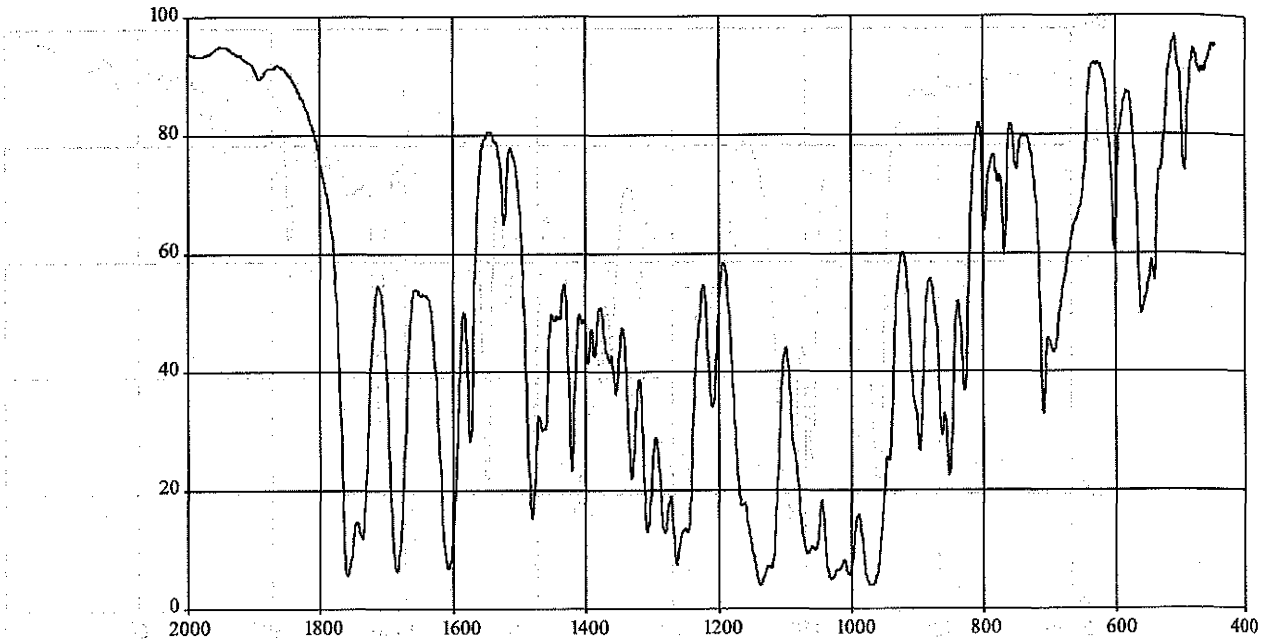
Adrenaline *Dispersive IR* Phase: KBr disc



Adefovir Dipivoxil

FTIR

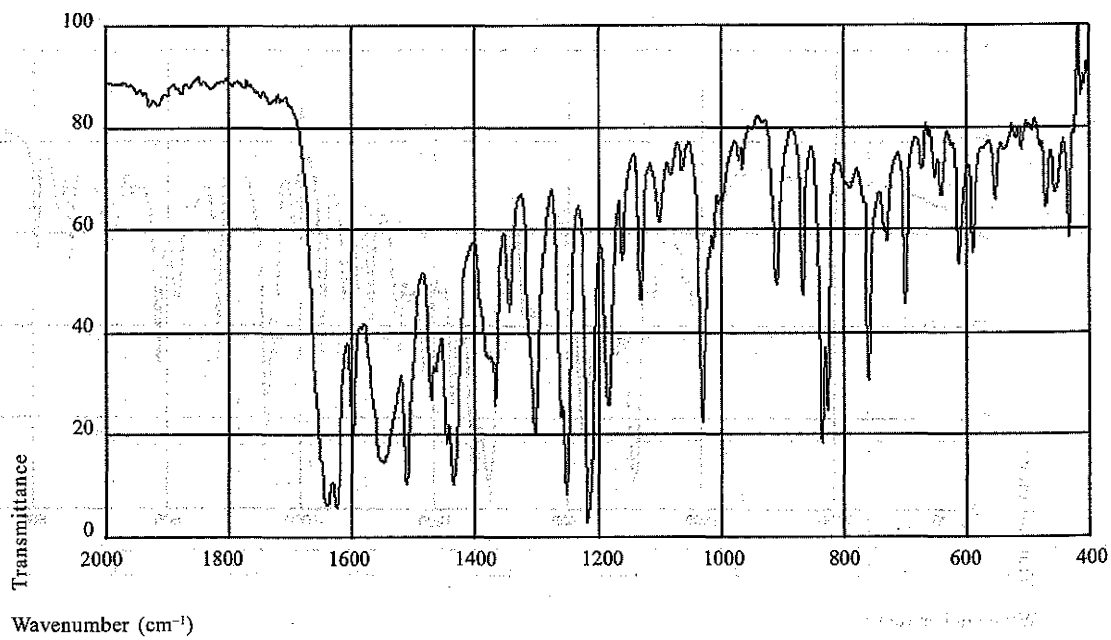
Phase: KBr disc



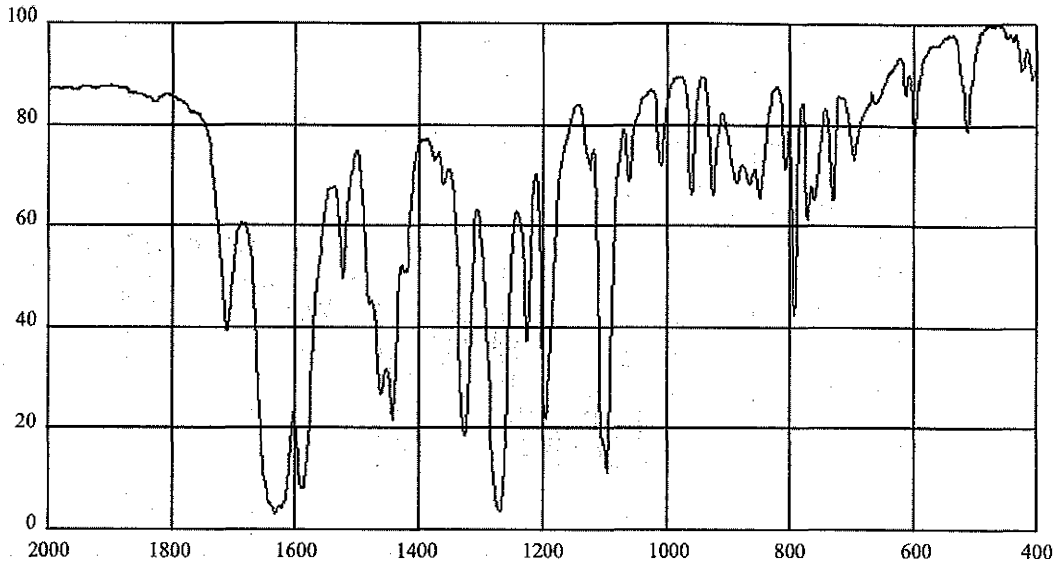
Agomelatine

FTIR

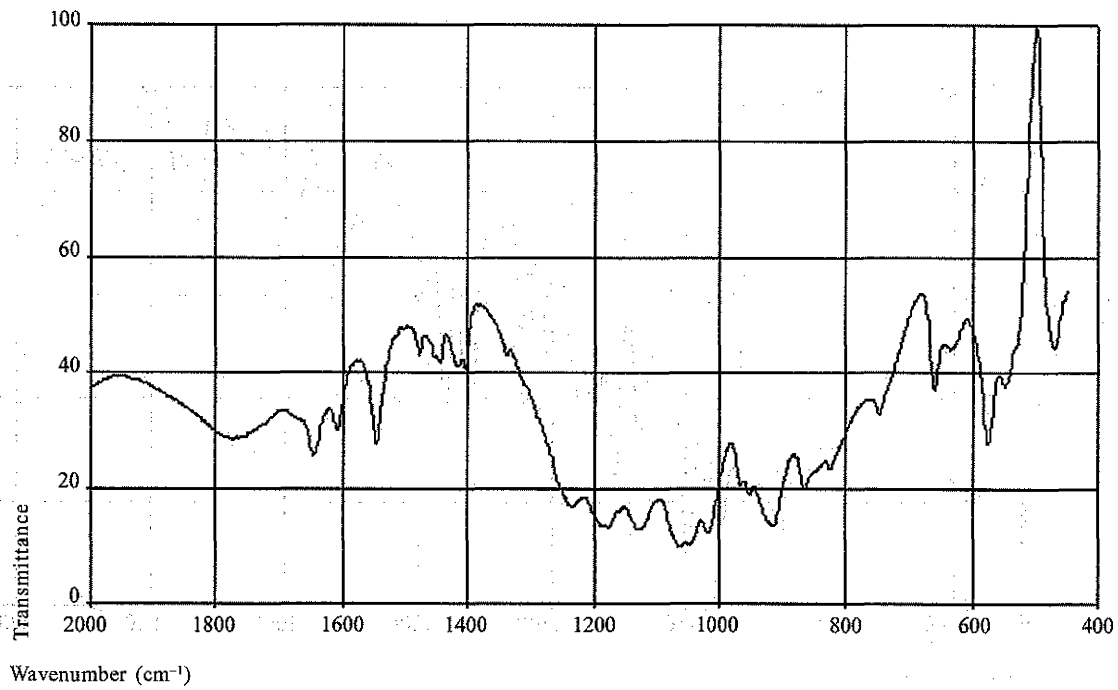
Phase: KBr disc



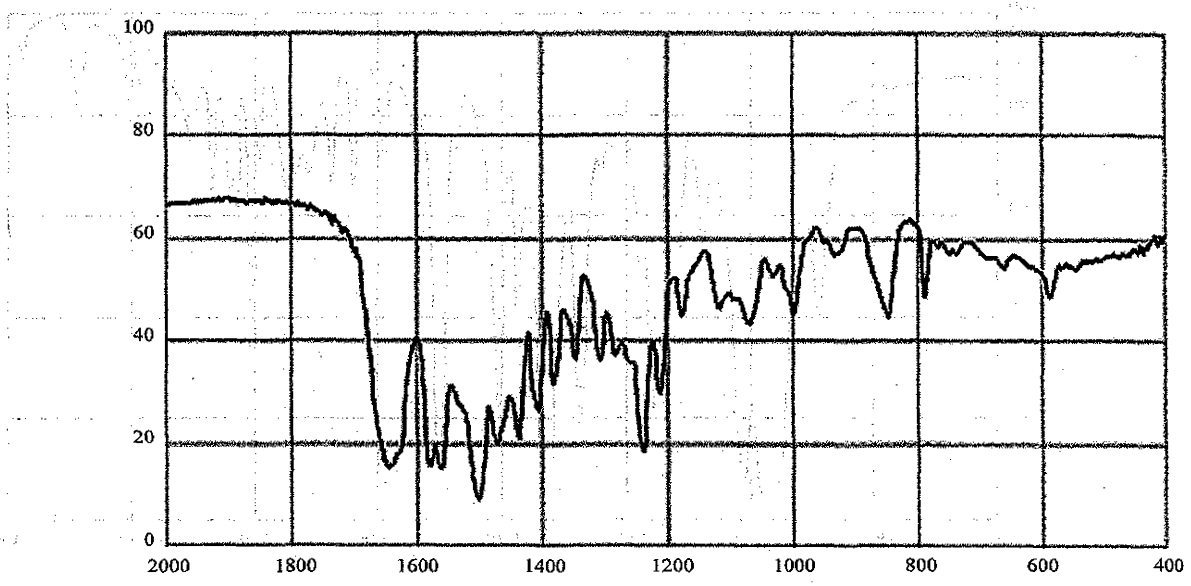
Albendazole *FTIR* Phase: KBr disc



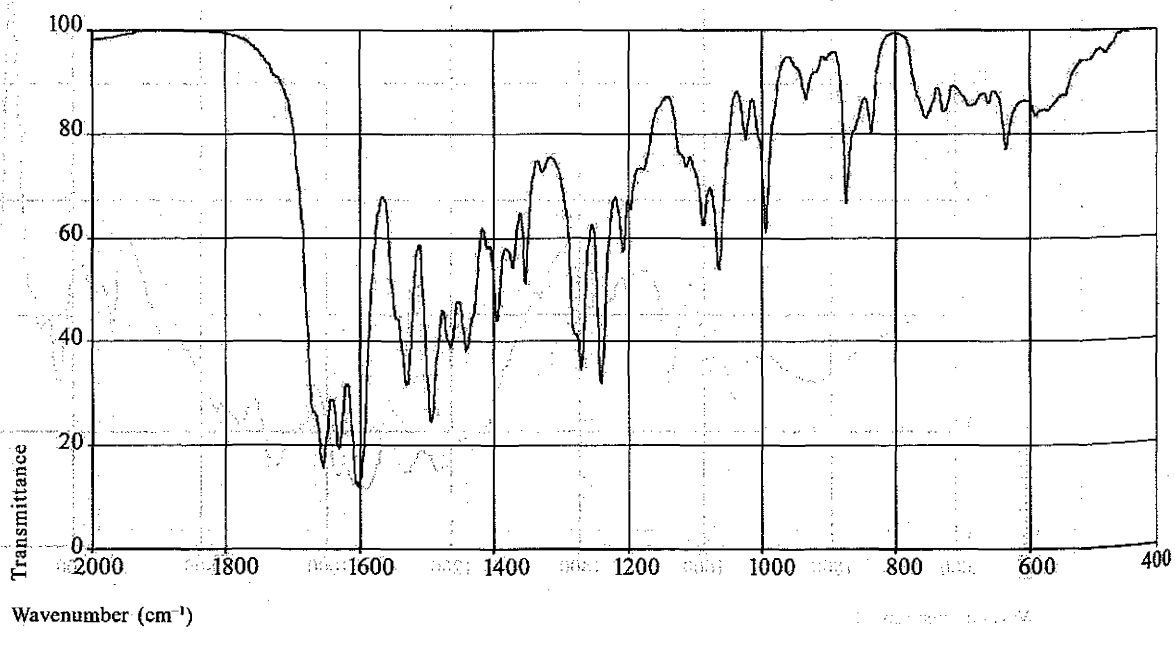
Alendronate Sodium *FTIR* Phase: KBr disc



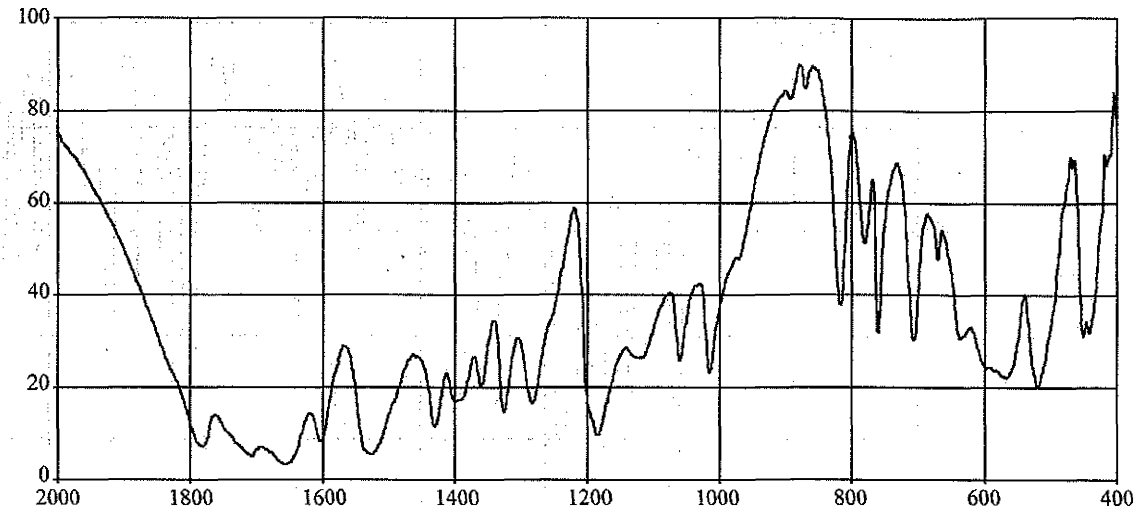
Alfuzosin *FTIR* Phase: KBr disc



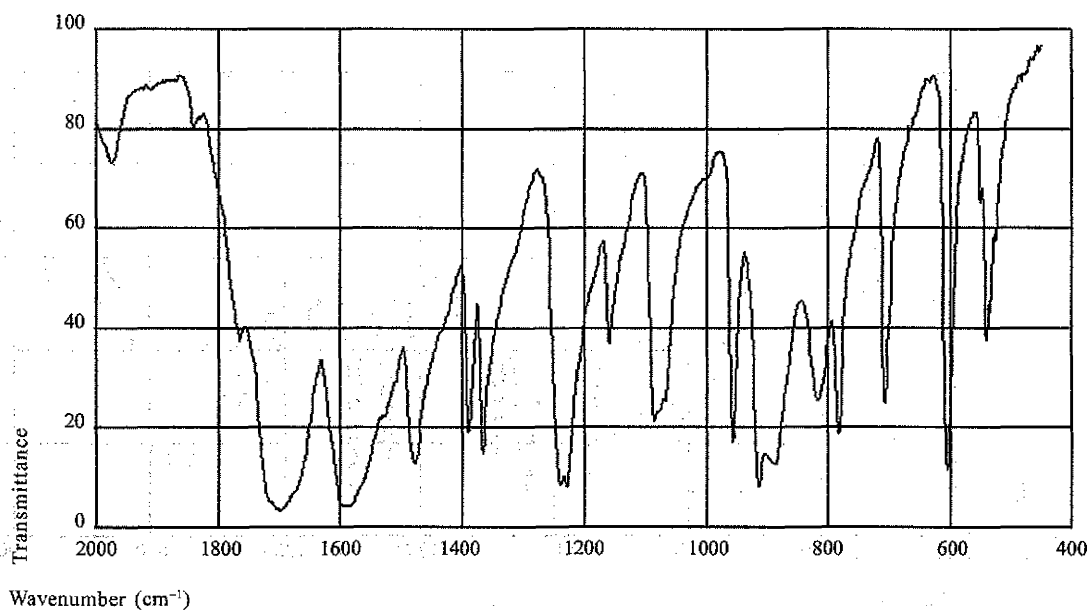
Alfuzosin Hydrochloride *FTIR* Phase: KBr disc



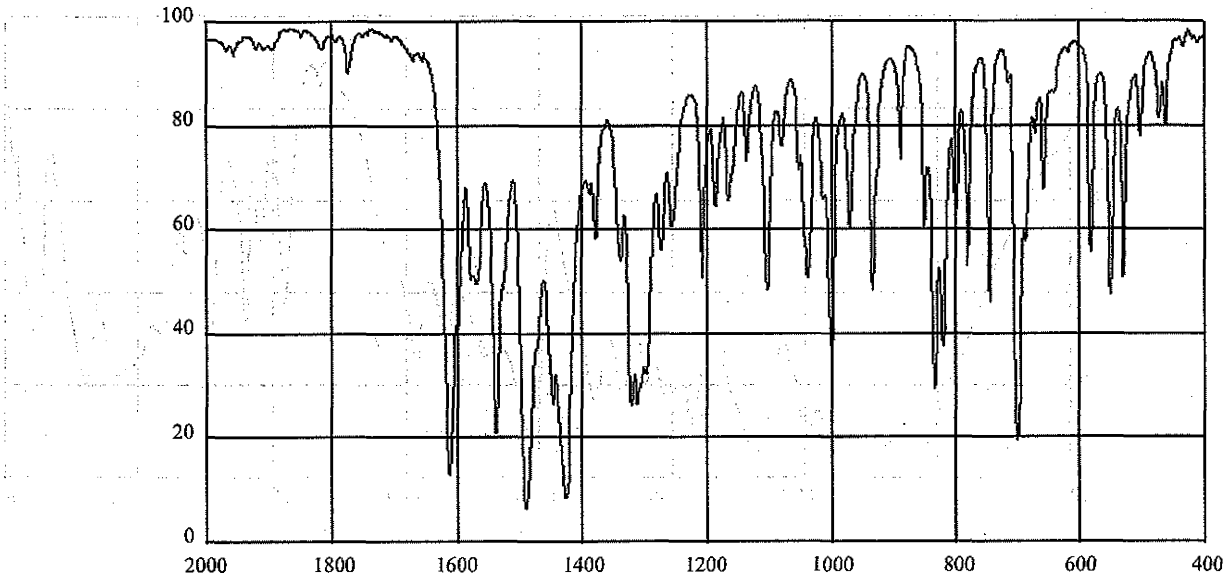
Allantoin *FTIR* Phase: KBr disc



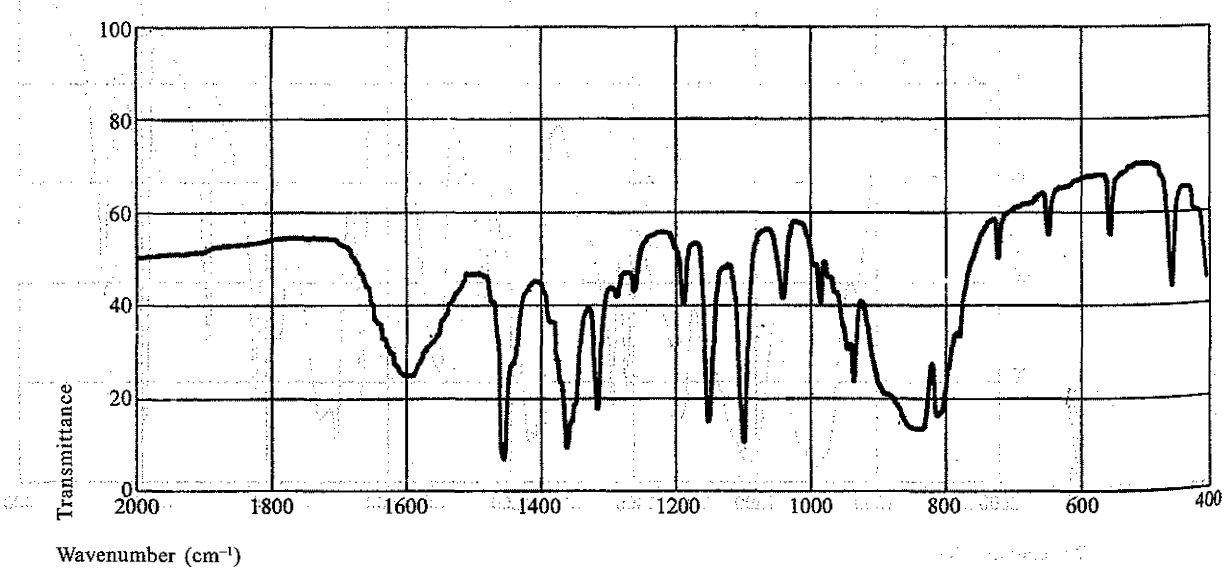
Allopurinol *FTIR* Phase: KBr disc



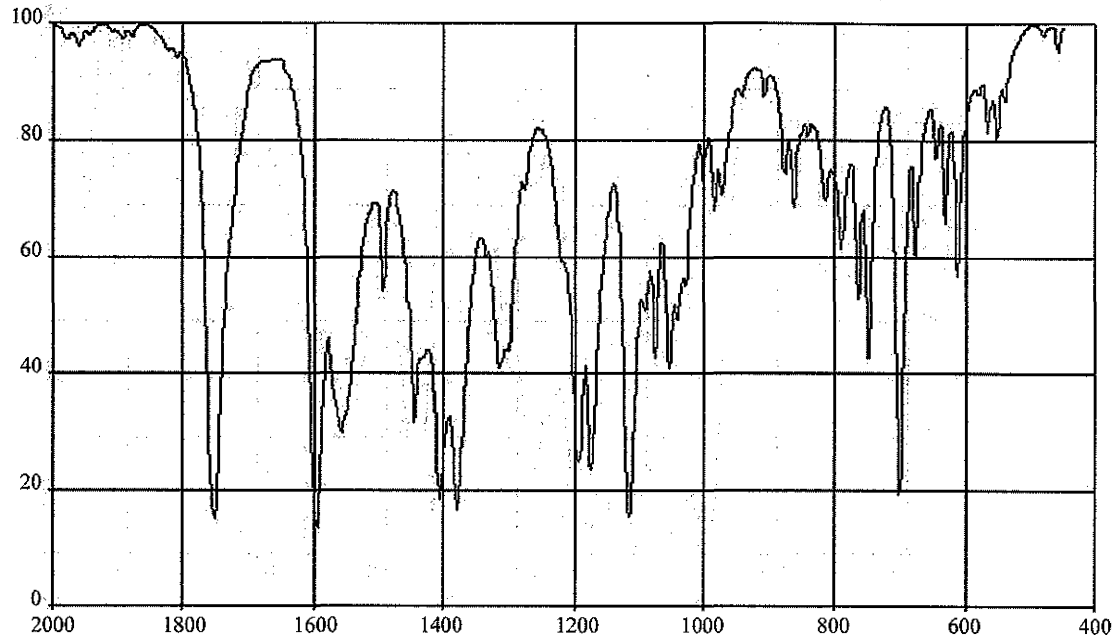
Alprazolam *FTIR* Phase: KBr disc



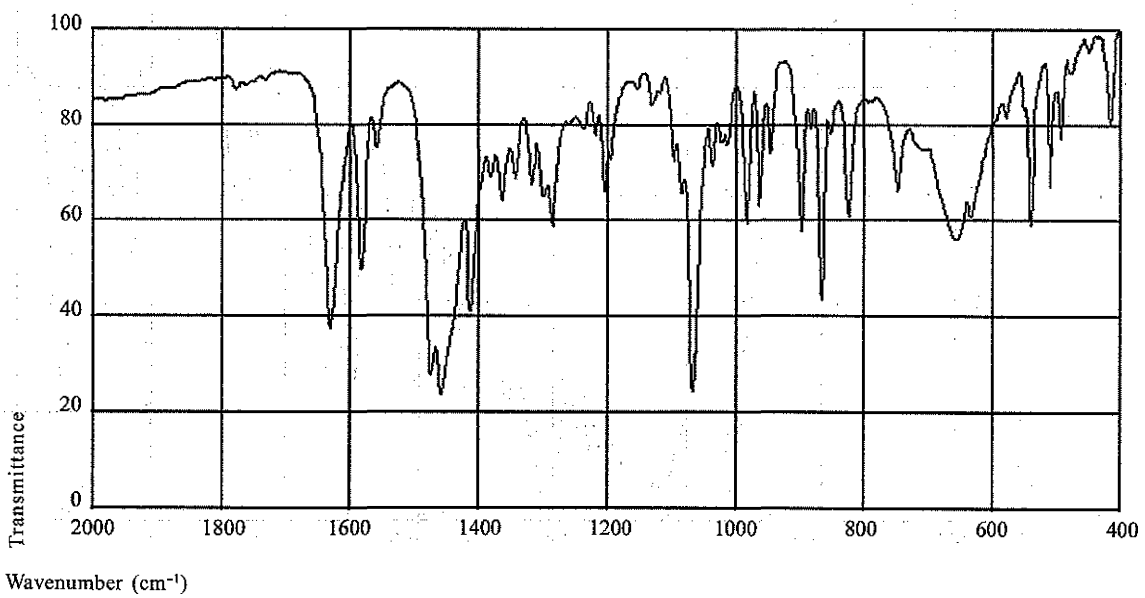
Amantadine *Dispersive IR* Phase: KBr disc



Ambrisentan *FTIR* Phase: KBr disc



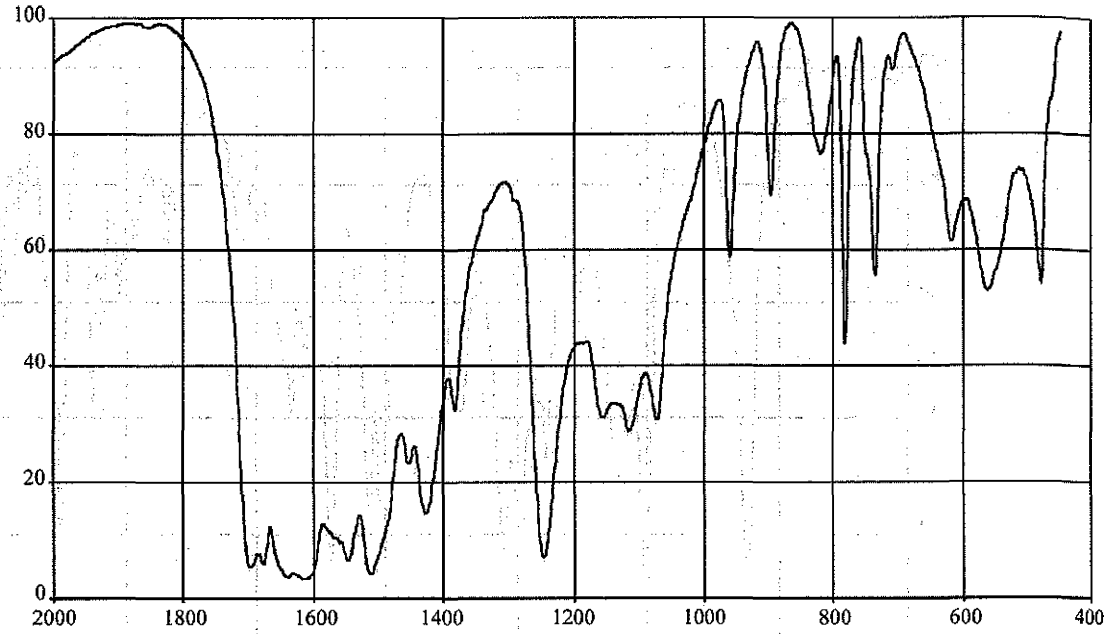
Ambroxol Hydrochloride *FTIR* Phase: KBr disc



Amiloride Hydrochloride

FTIR

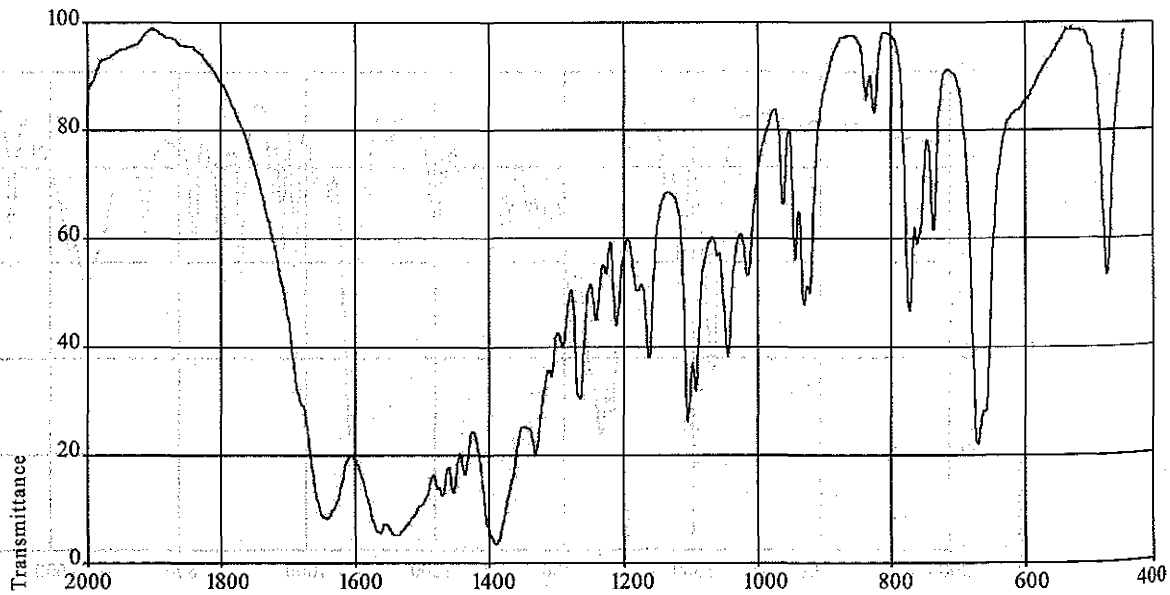
Phase: KCl disc



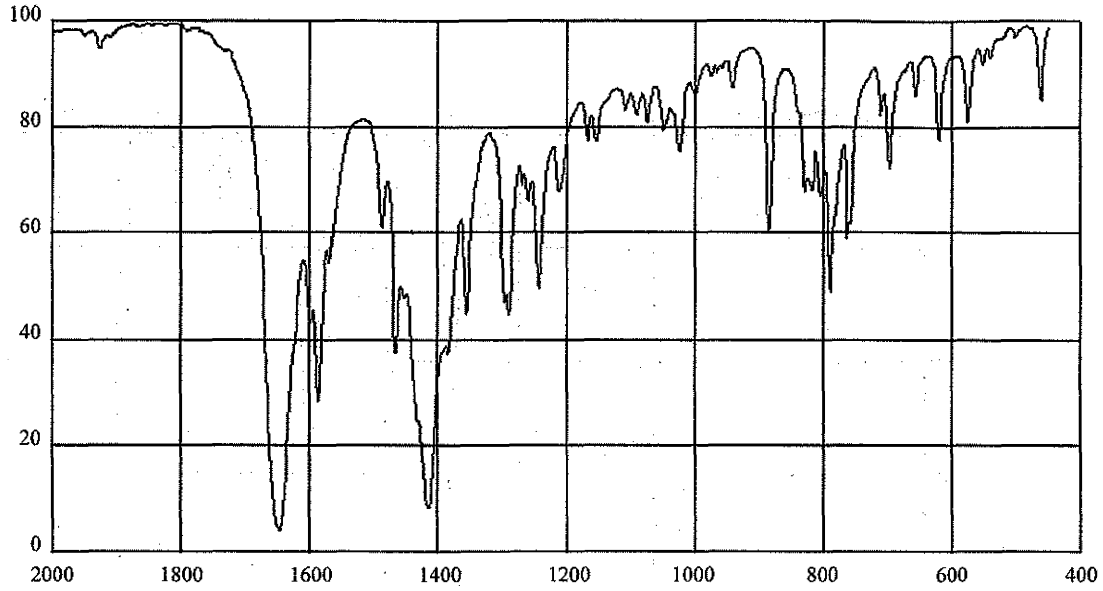
Aminocaproic Acid

FTIR

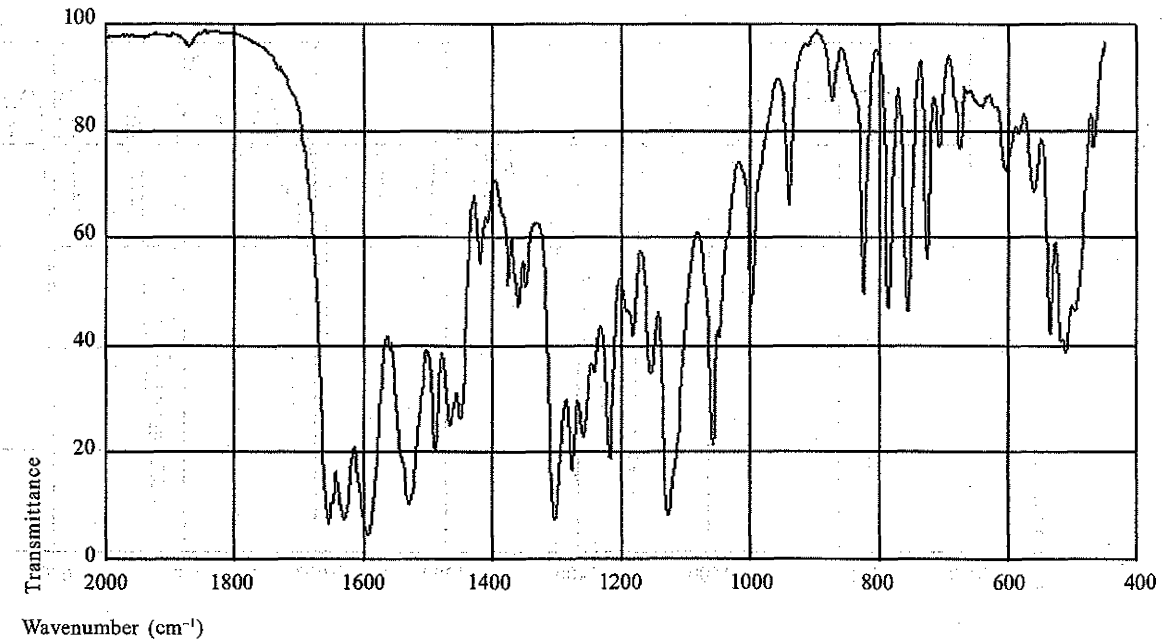
Phase: KBr disc



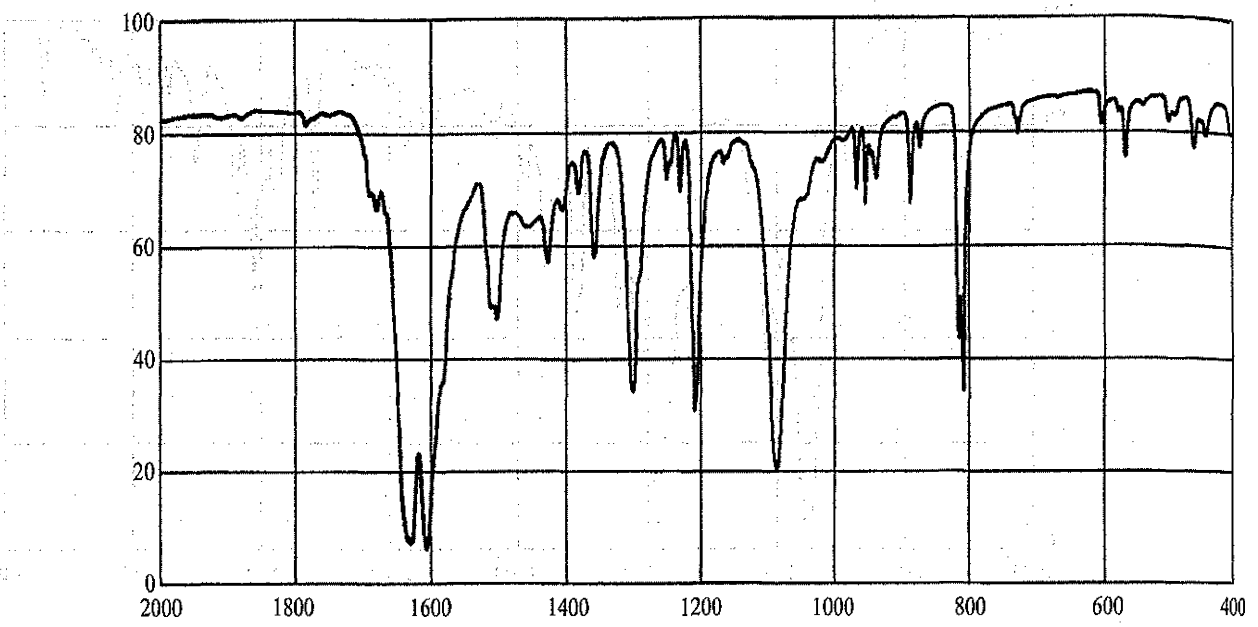
Amiodarone Hydrochloride *FTIR* Phase: KBr disc



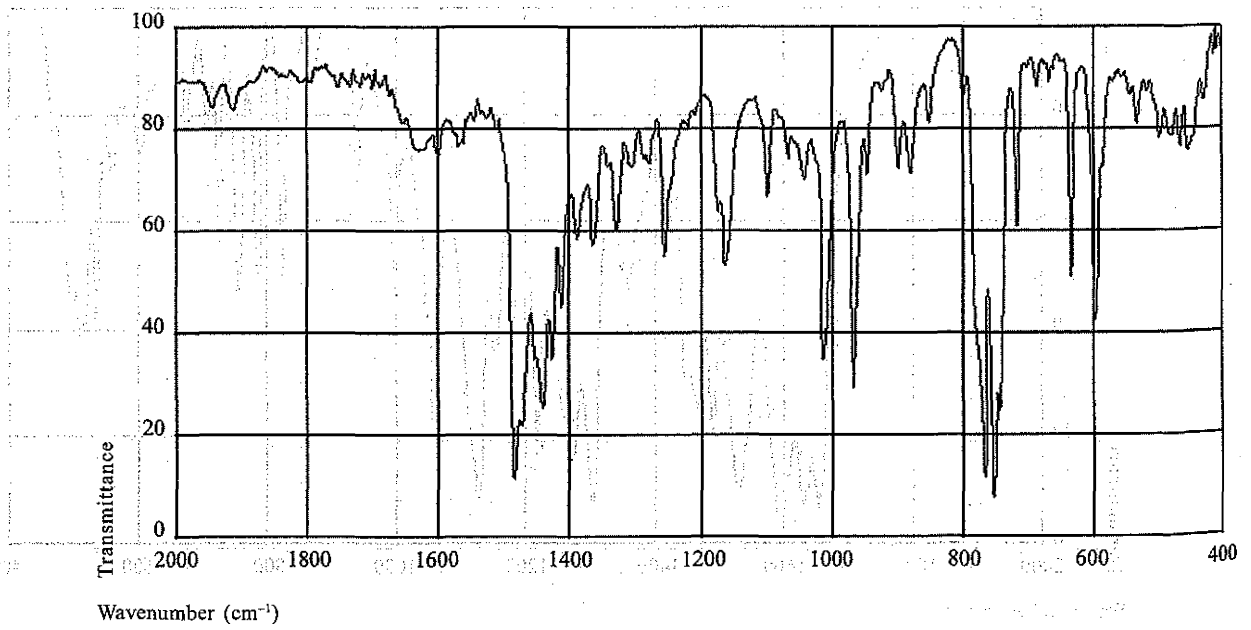
Amisulpride *FTIR* Phase: KBr disc



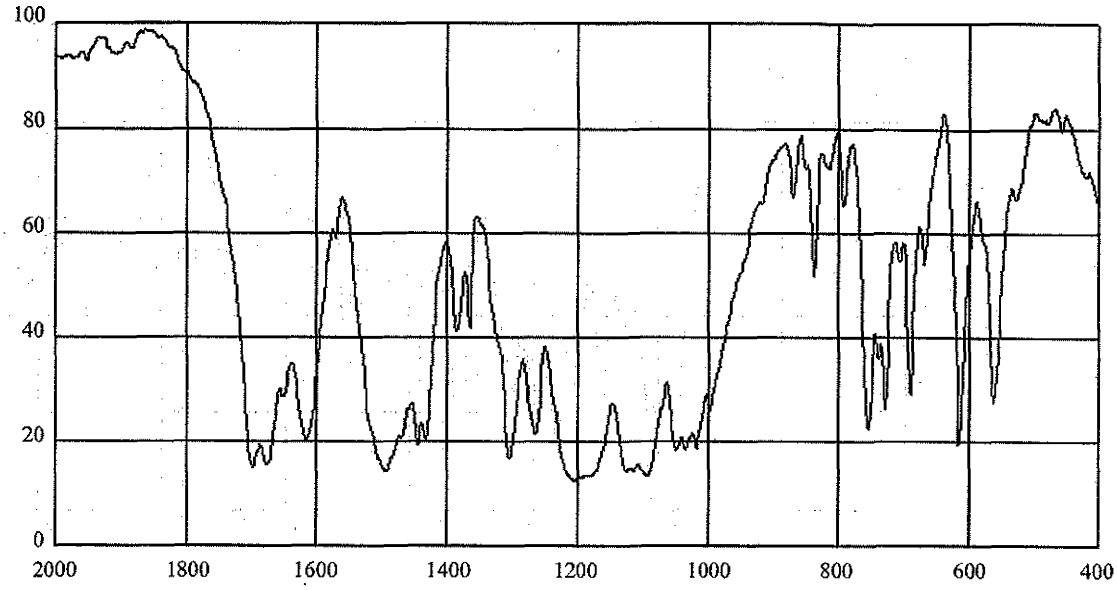
Amitraz *Dispersive IR* Phase: KBr disc



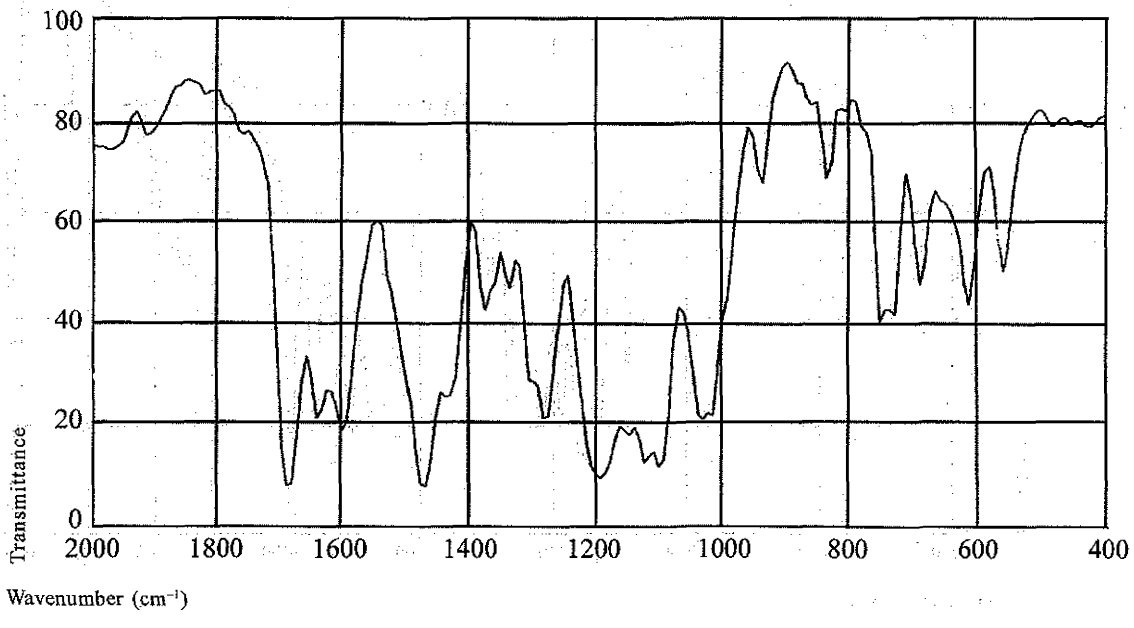
Amitriptyline Hydrochloride *FTIR* Phase: KBr disc



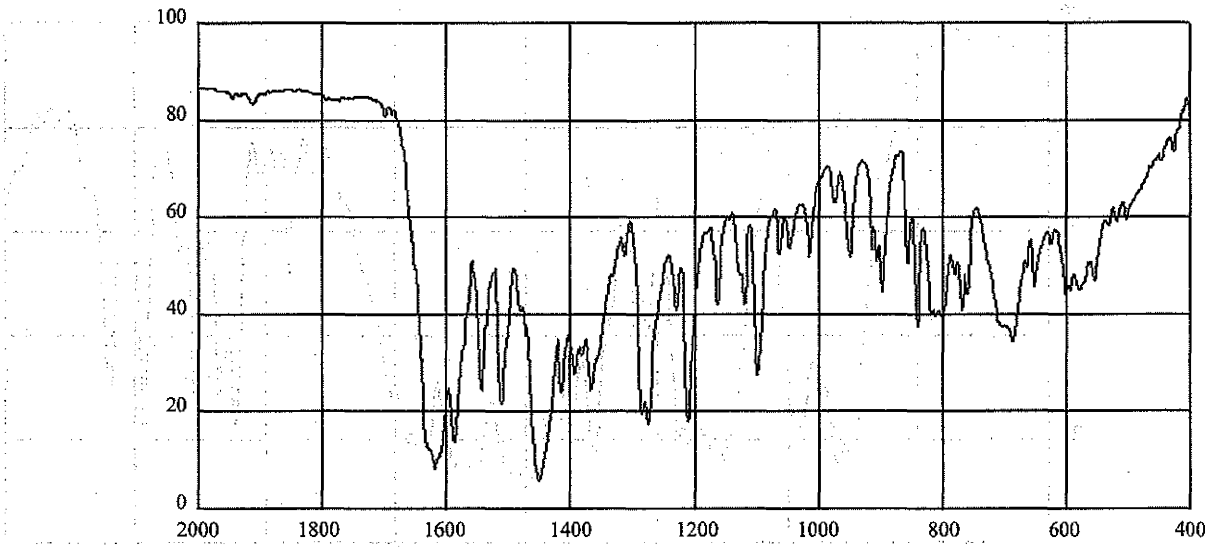
Amlodipine Besylate *FTIR* Phase: Liquid paraffin mull



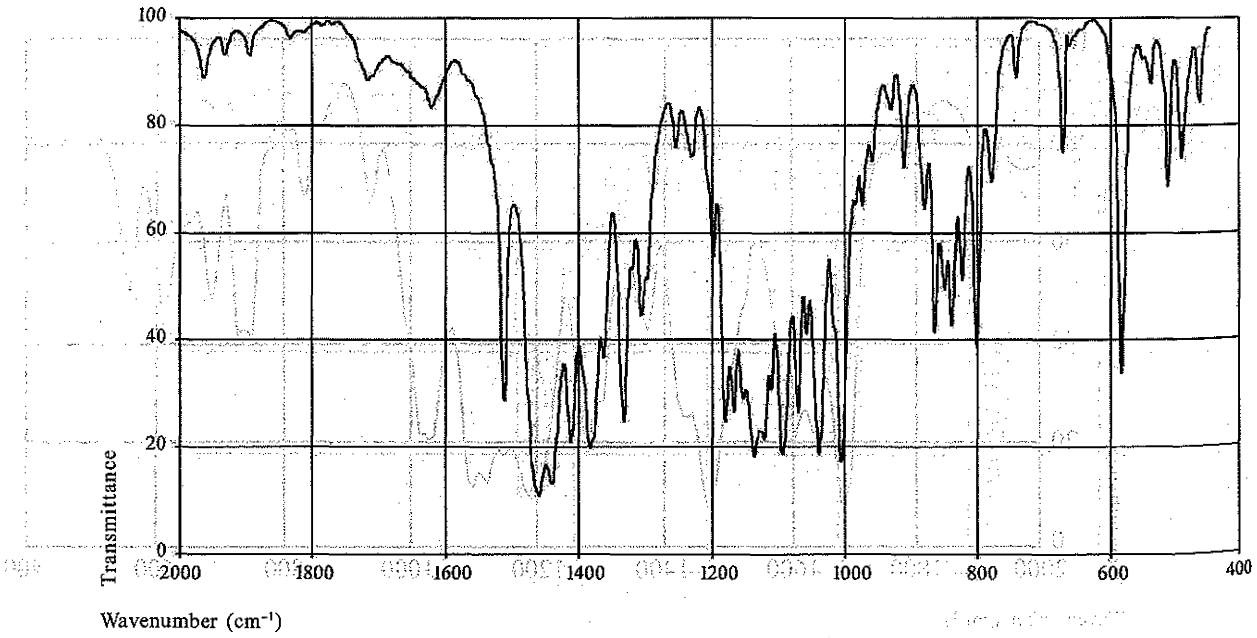
***S*-Amlodipine Besylate** *FTIR* Phase: Liquid paraffin mull



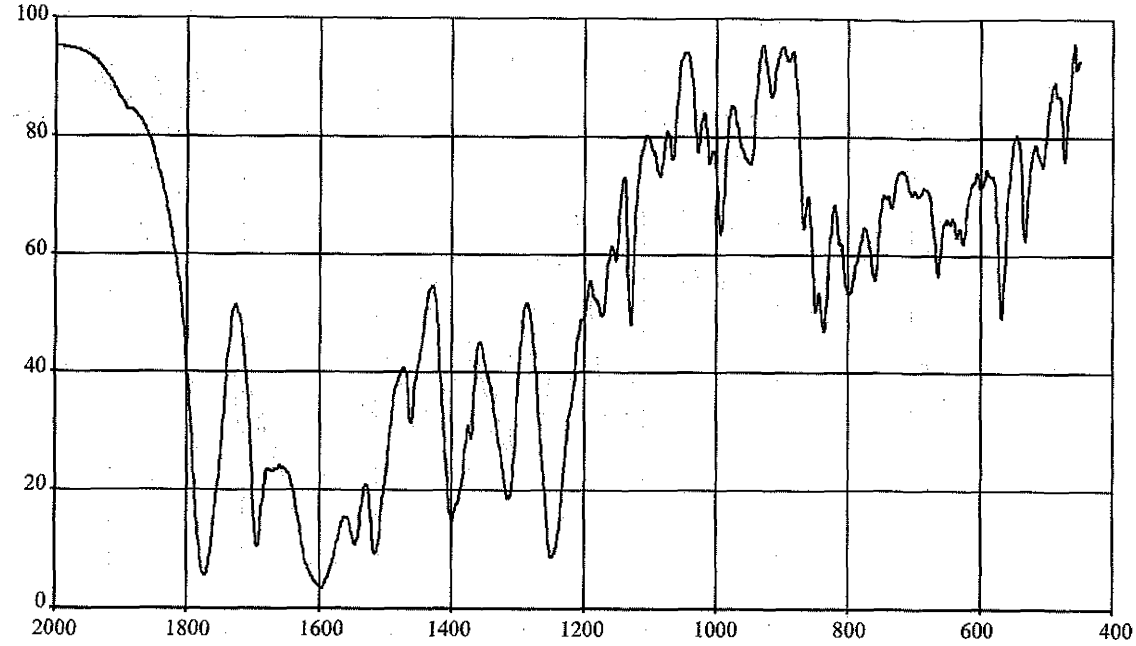
Amodiaquine *FTIR* Phase: KBr disc



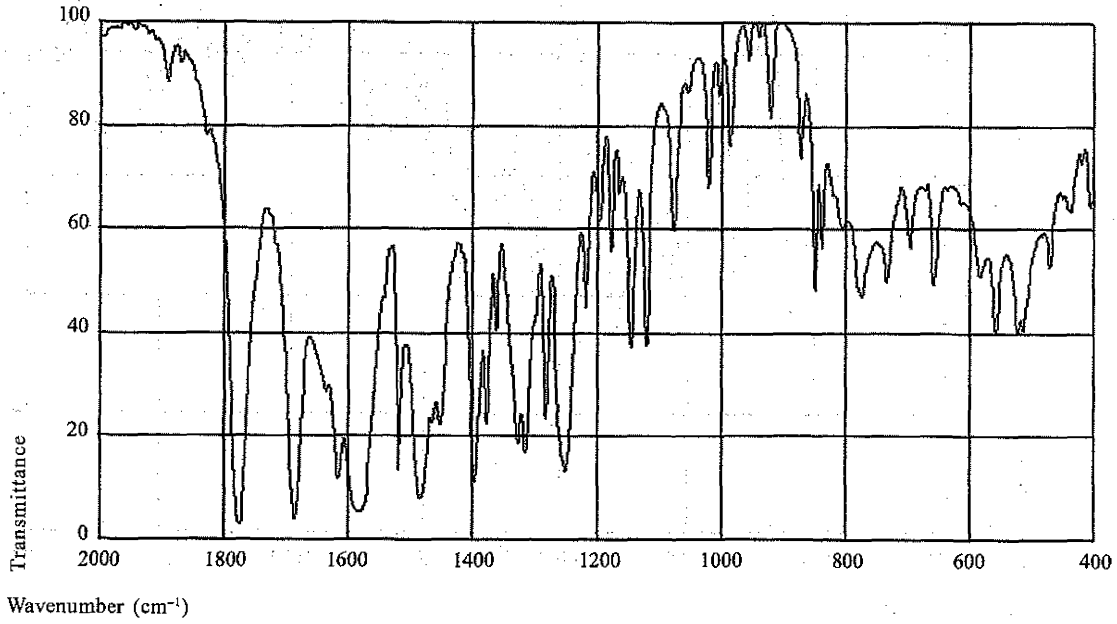
Amorolfine Hydrochloride *FTIR* Phase: KBr disc



Amoxycillin Sodium *FTIR* Phase: KBr disc



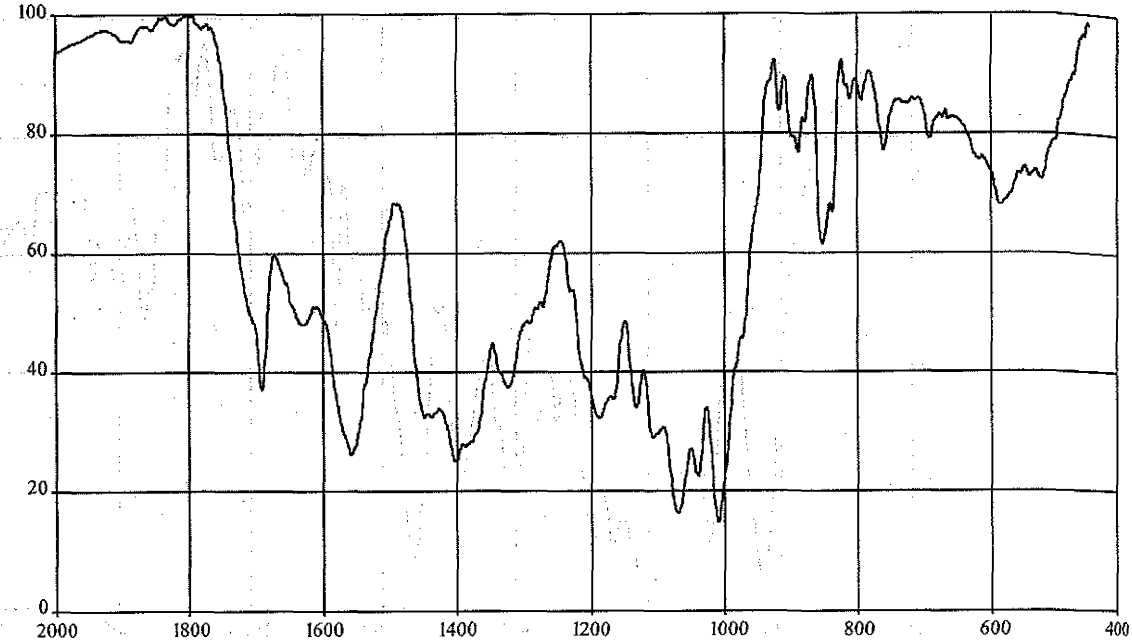
Amoxycillin Trihydrate *FTIR* Phase: KBr disc



Amphotericin B

FTIR

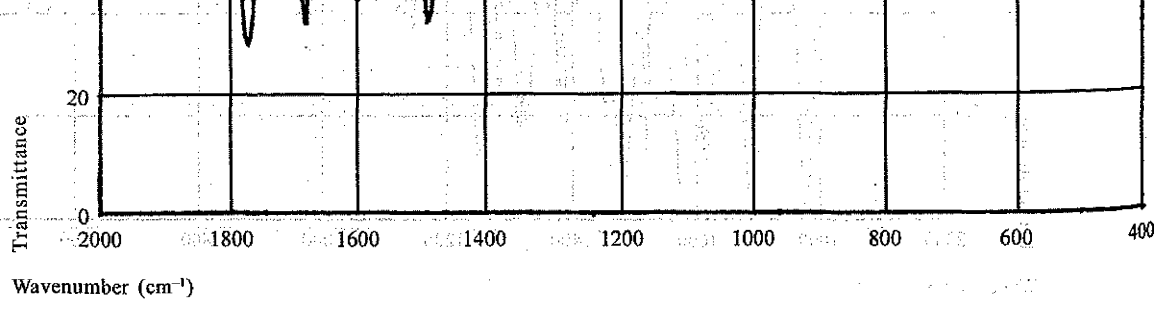
Phase: KBr disc



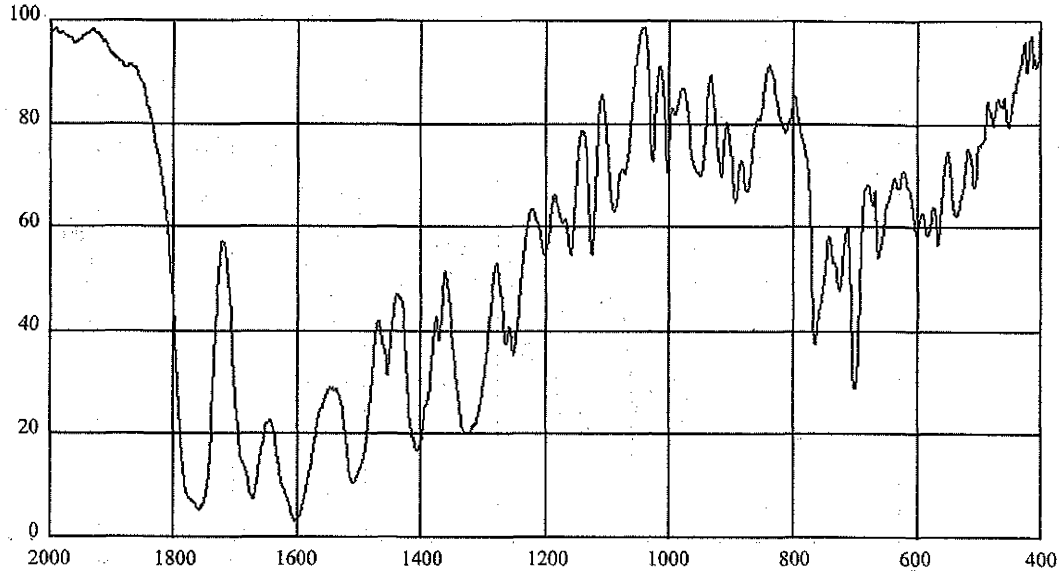
Ampicillin

FTIR

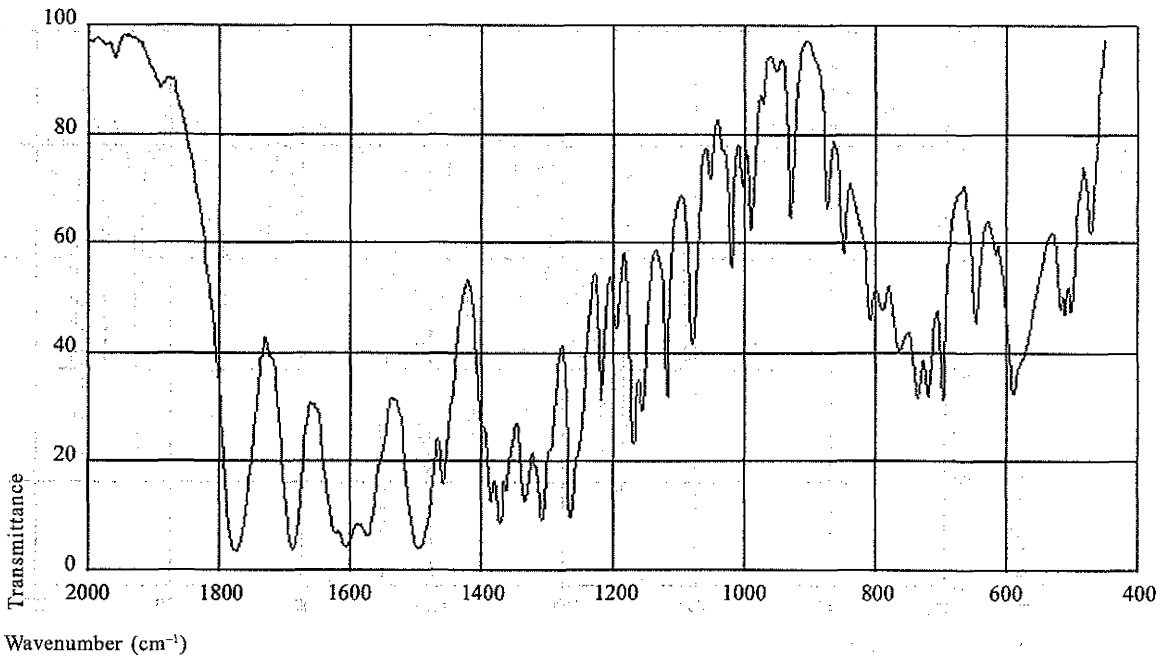
Phase: KBr disc



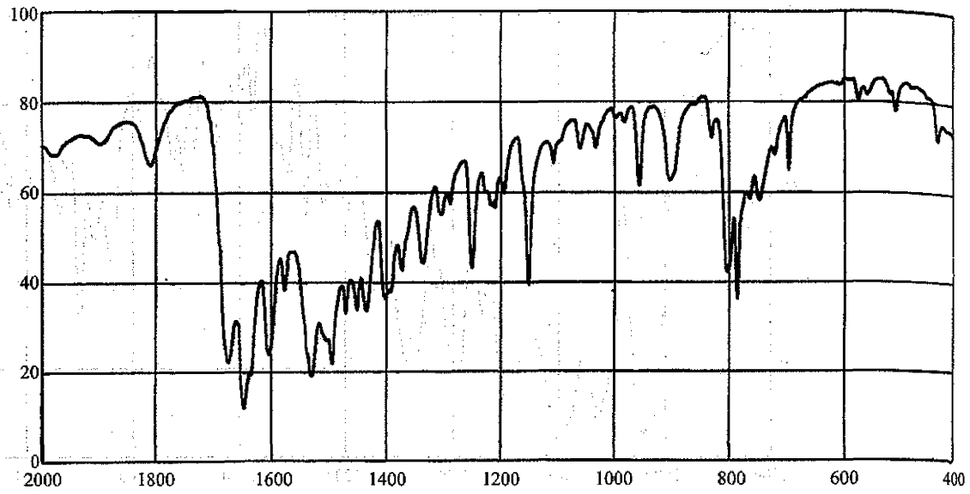
Ampicillin Sodium *FTIR* Phase: KBr disc



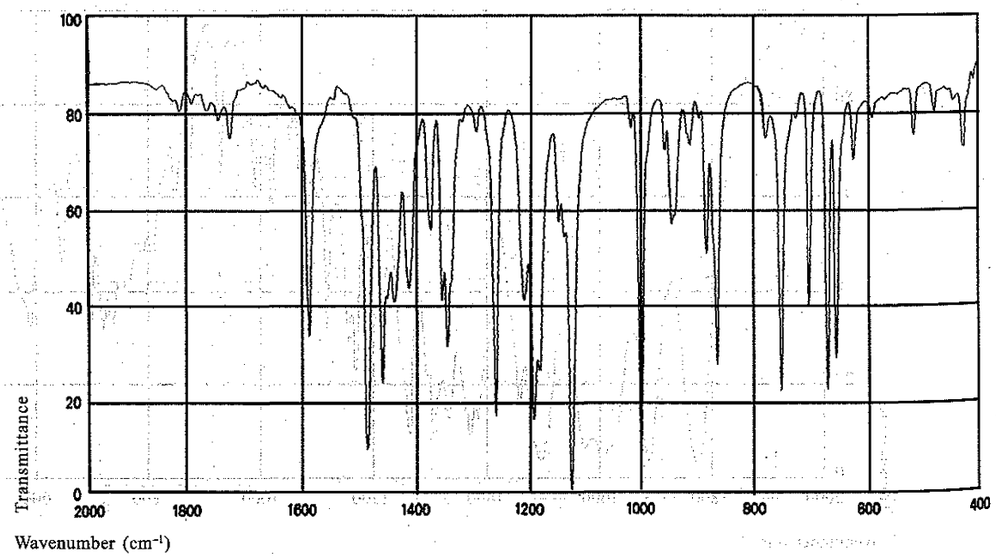
Ampicillin Trihydrate *FTIR* Phase: KBr disc



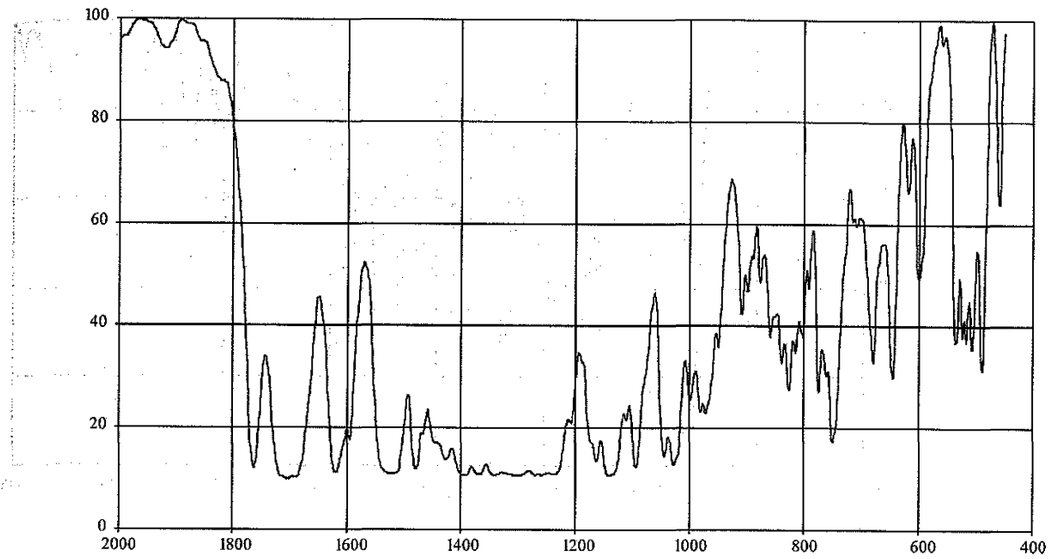
Amprolium Hydrochloride *Dispersive IR* Phase: KCl disc



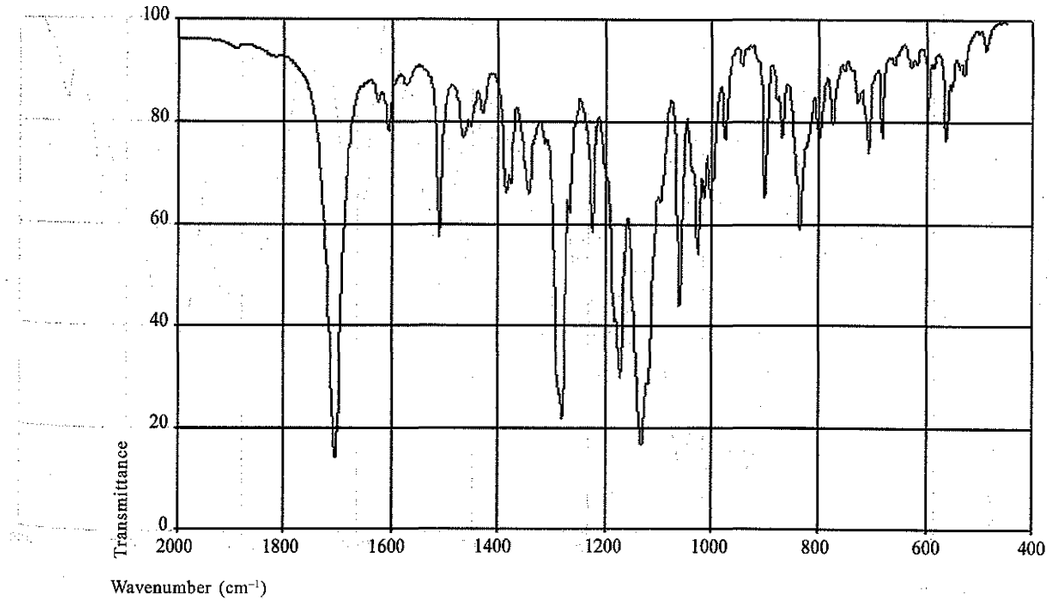
Anastrozole *FTIR* Phase: KBr disc



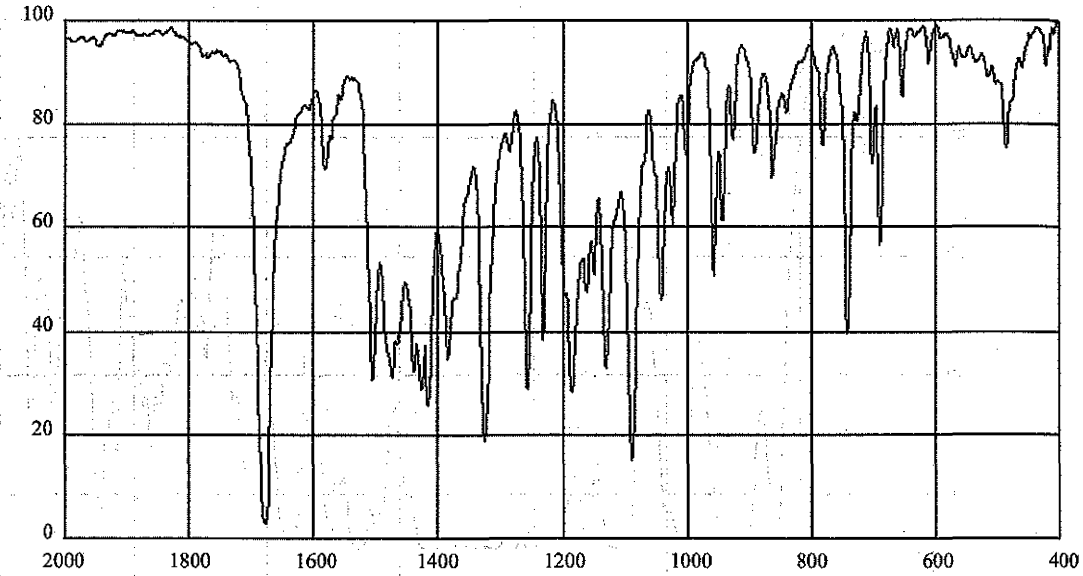
Apremilast *FTIR* Phase: KBr disc



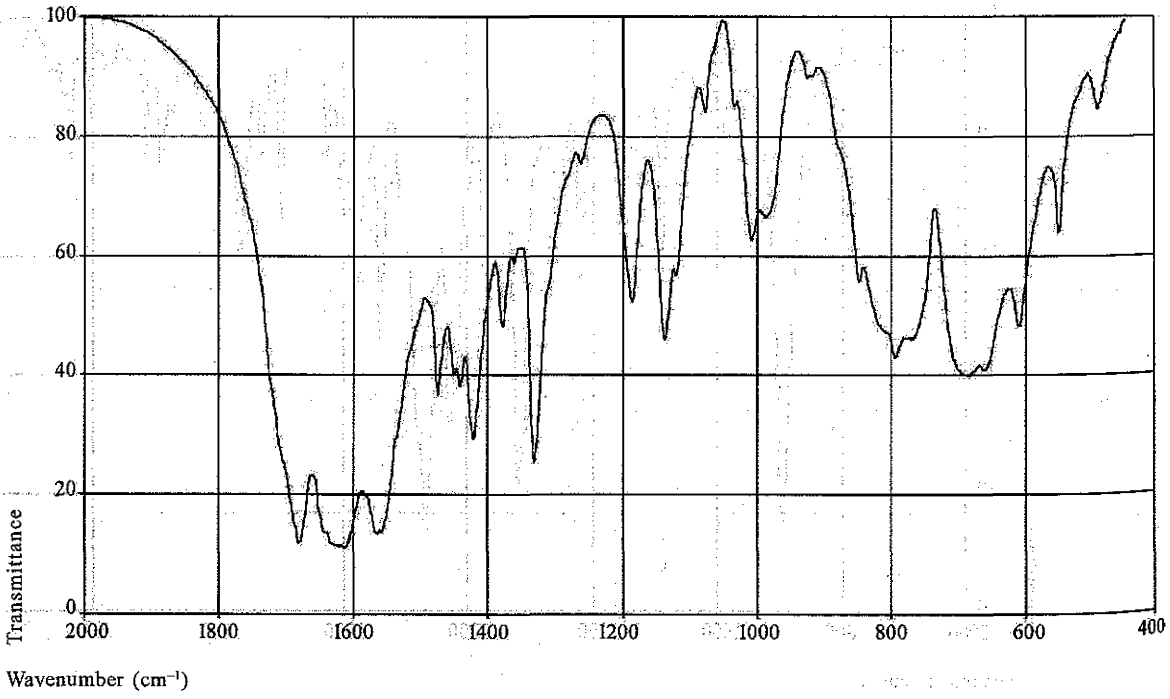
Aprepitant *FTIR* Phase: KBr disc



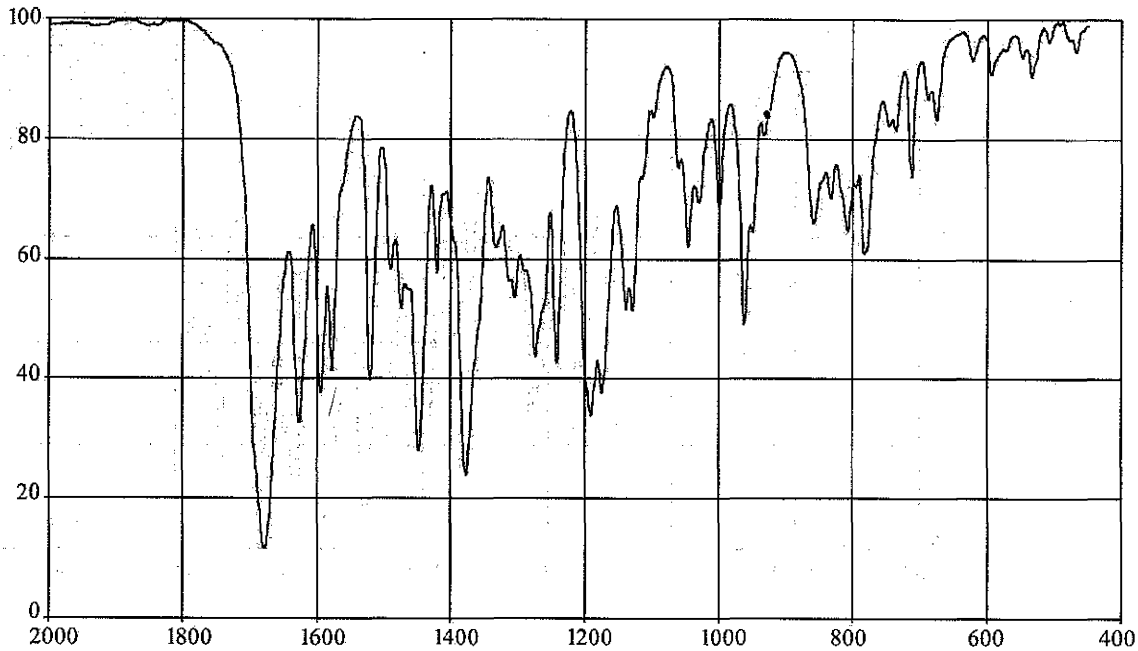
Arbidol Hydrochloride *FTIR* Phase: KBr disc



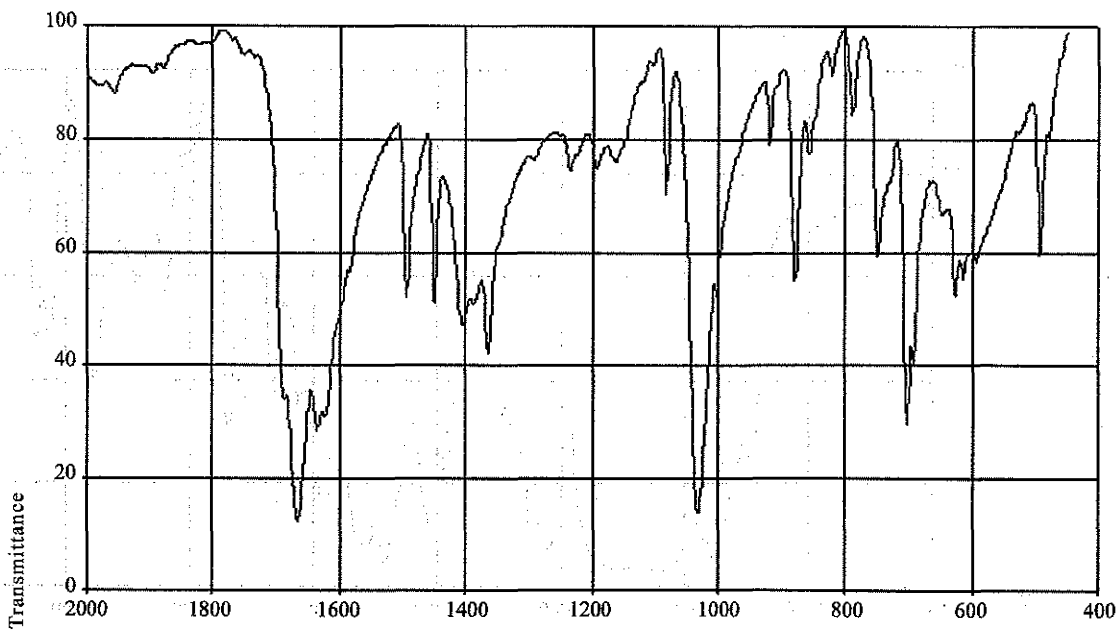
Arginine *FTIR* Phase: KBr disc



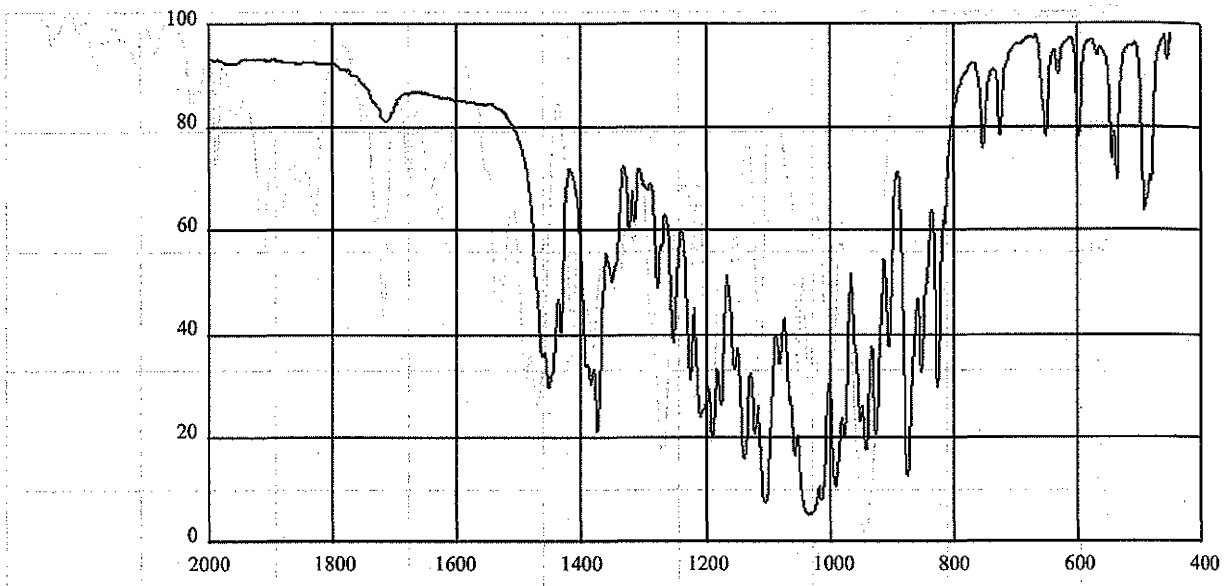
Aripiprazole *FTIR* Phase: KBr disc



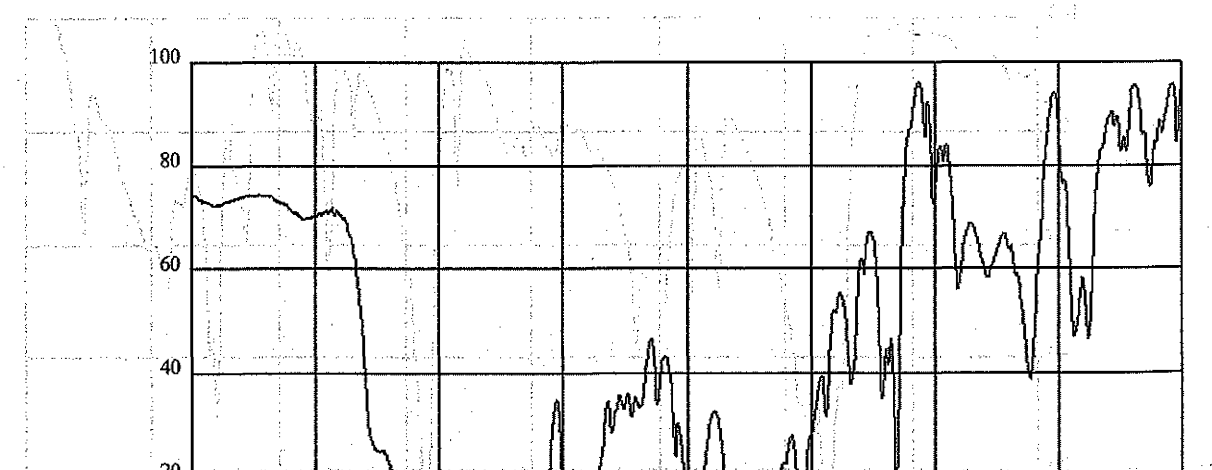
Armodafinil *FTIR* Phase: KBr disc



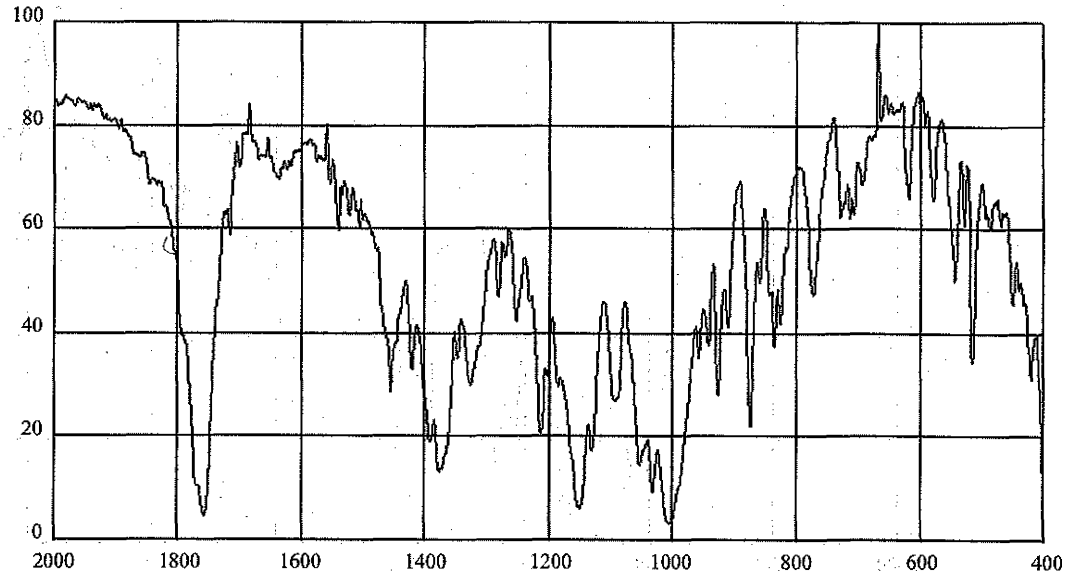
Artemether *FTIR* Phase: KBr disc



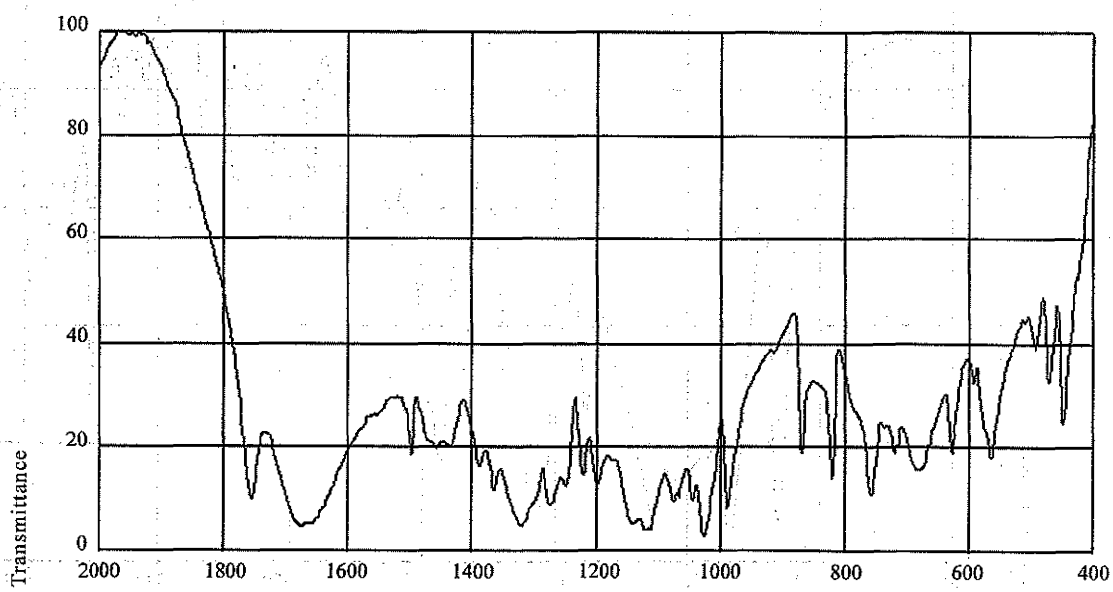
Arterolane Maleate *FTIR* Phase: KBr disc



Artesunate *FTIR* Phase: KBr disc



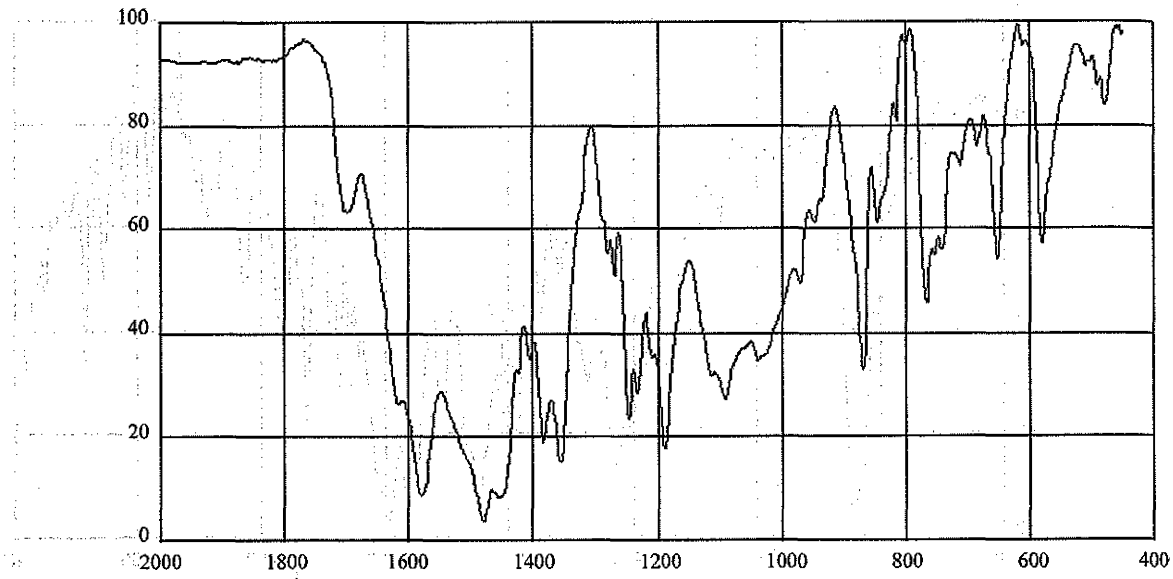
Ascorbic Acid *FTIR* Phase: KBr disc



Asenapine Maleate

FTIR

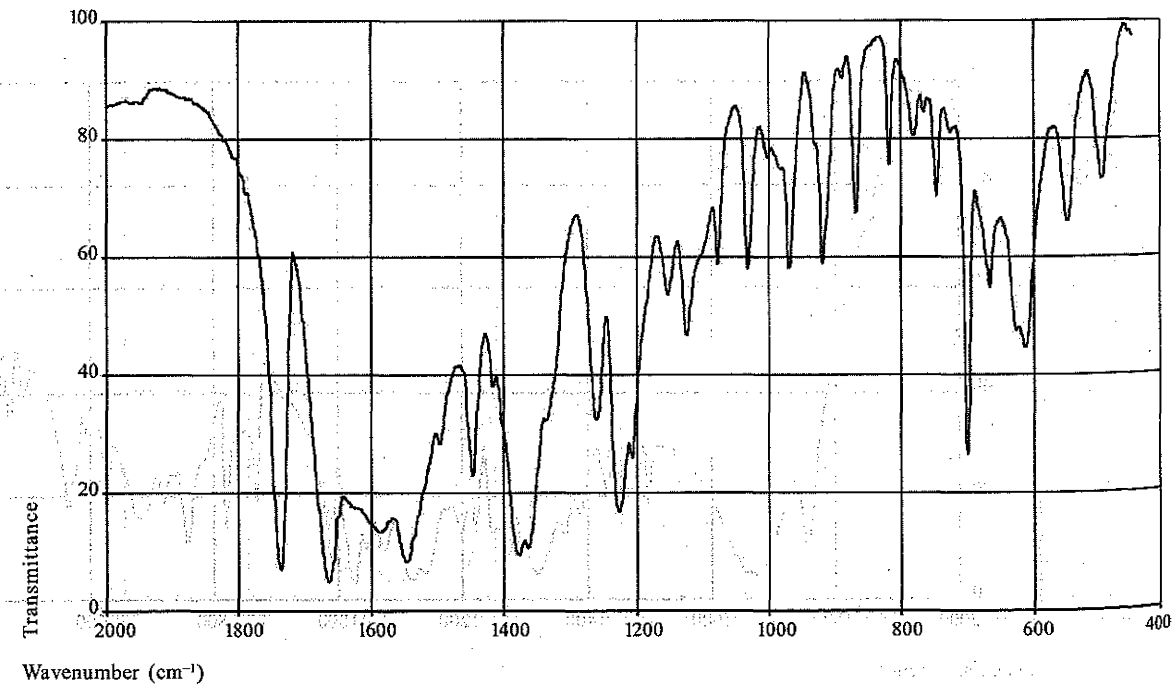
Phase: KBr disc



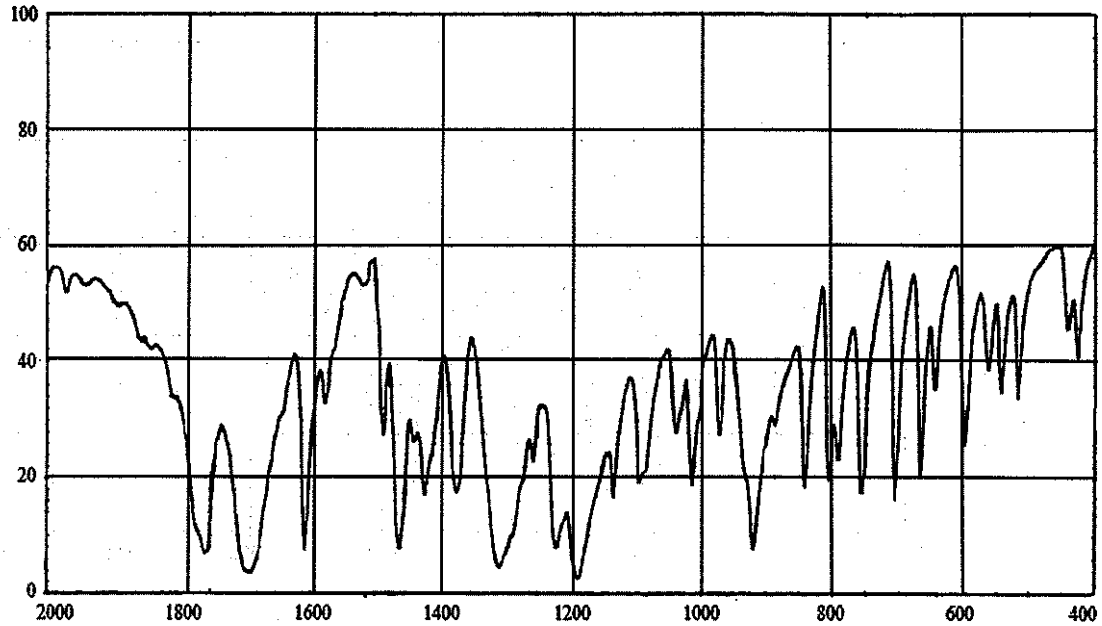
Aspartame

FTIR

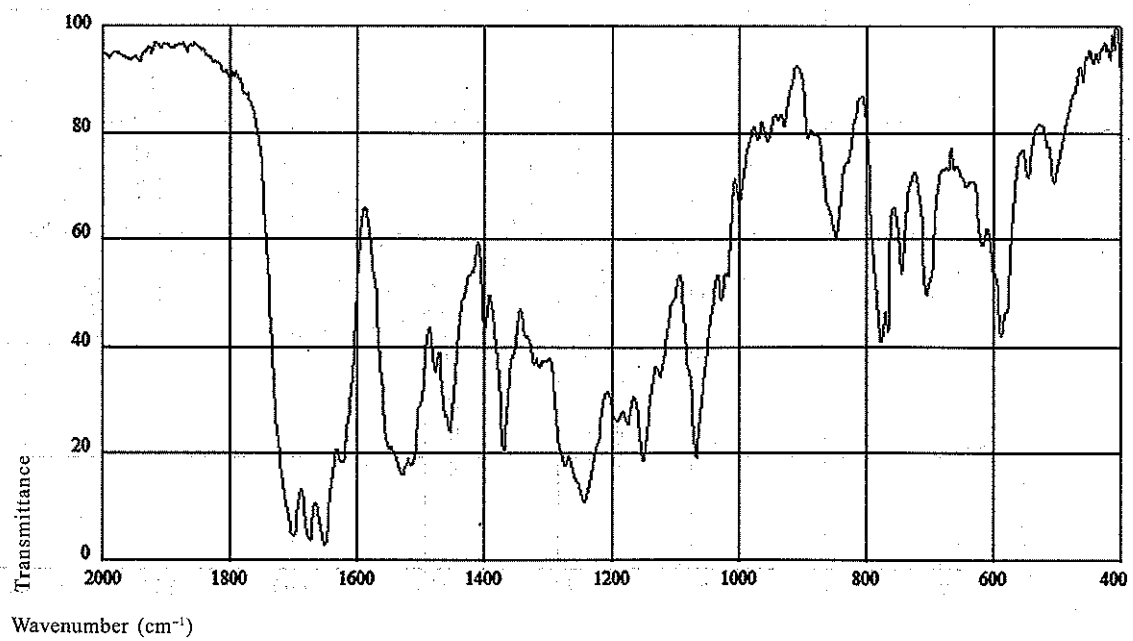
Phase: KBr disc



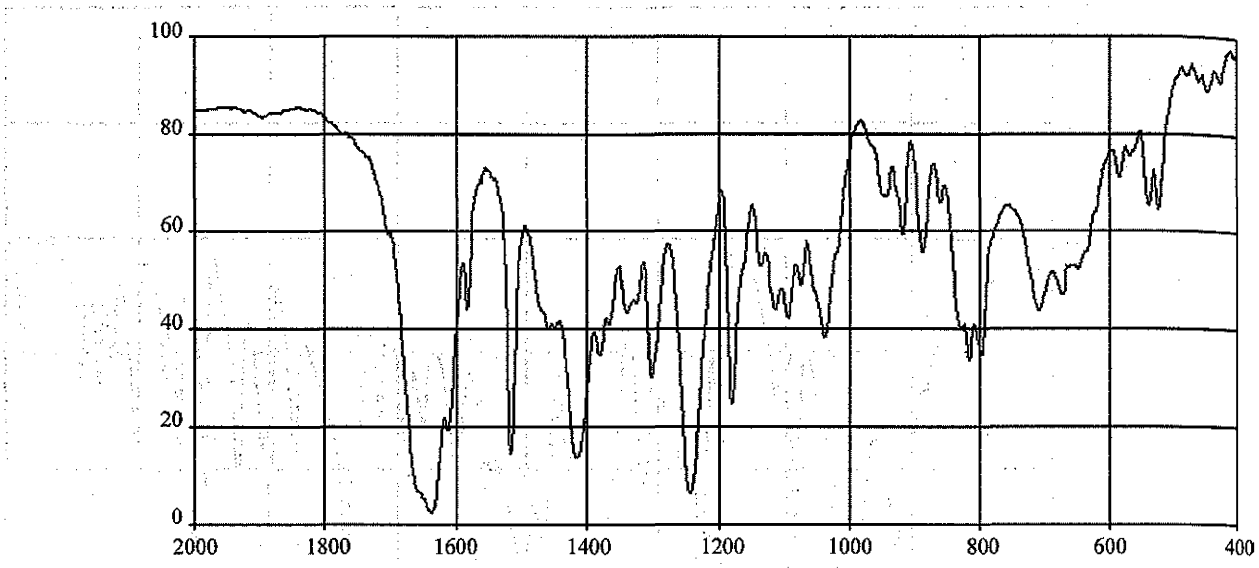
Aspirin *FTIR* Phase: KBr disc



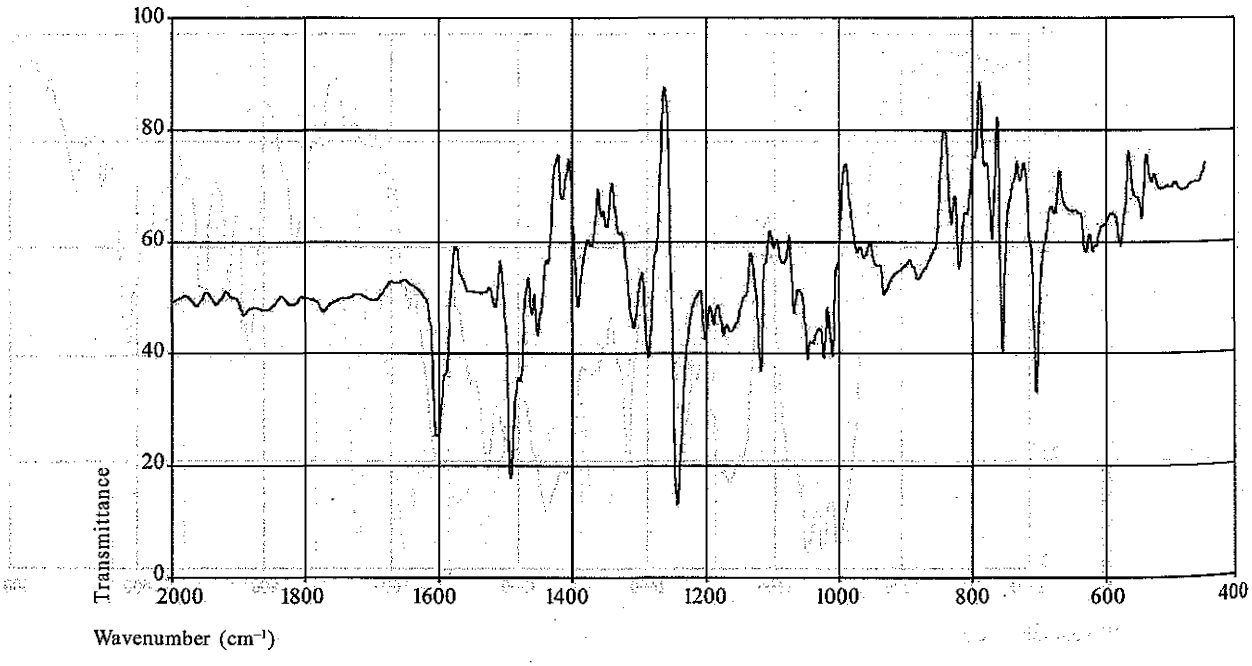
Atazanavir Sulphate *FTIR* Phase: KBr disc



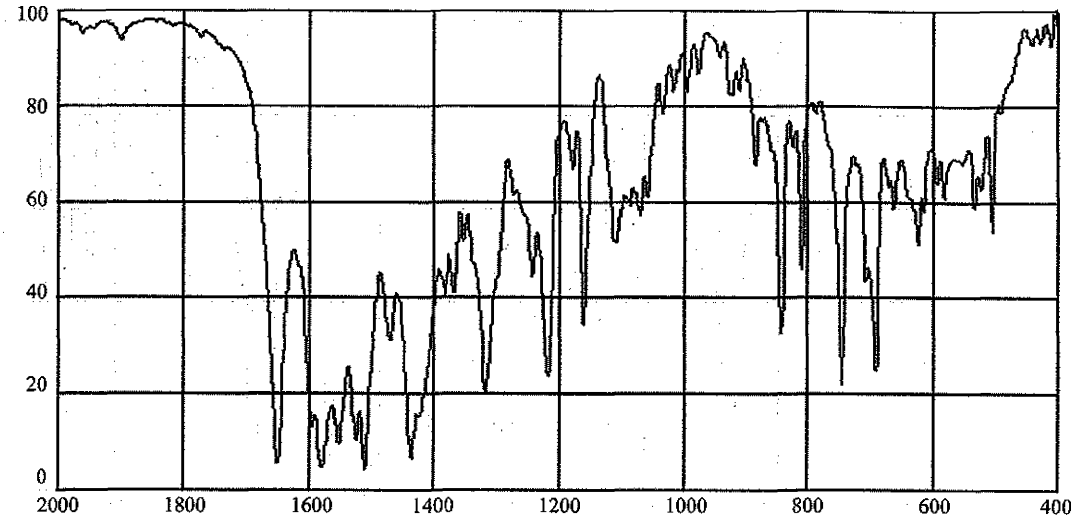
Atenolol *FTIR* Phase: KBr disc



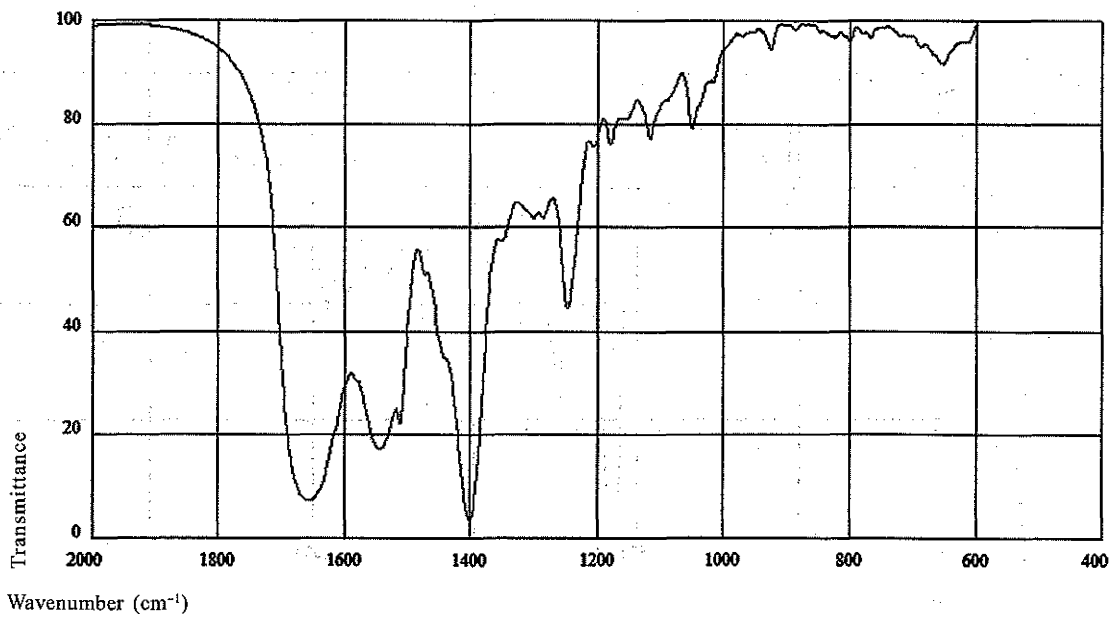
Atomoxetine Hydrochloride *FTIR* Phase: KBr disc



Atorvastatin Calcium *FTIR* Phase: KBr disc



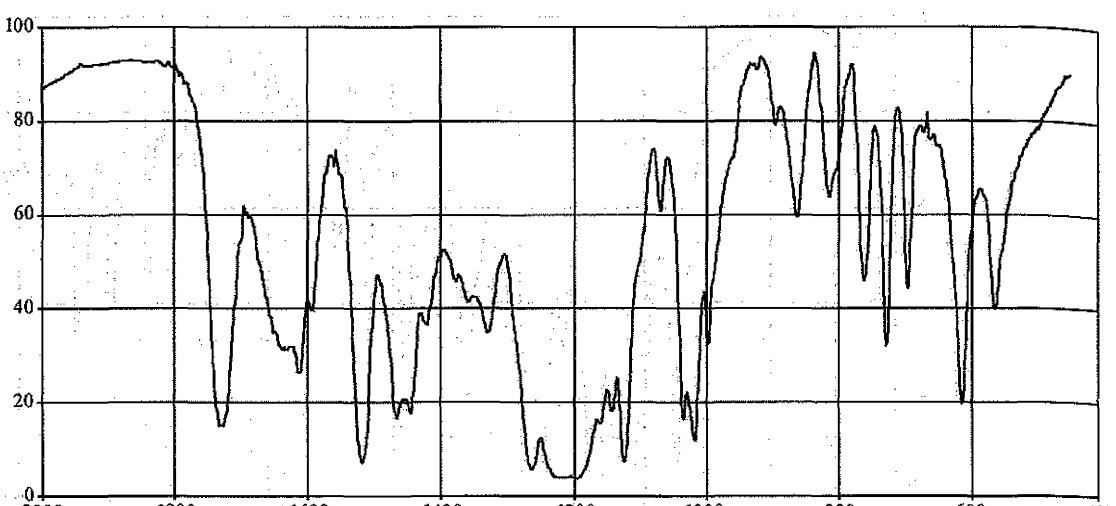
Atosiban Acetate *FTIR* Phase: KBr disc



Atracurium Besylate

FTIR

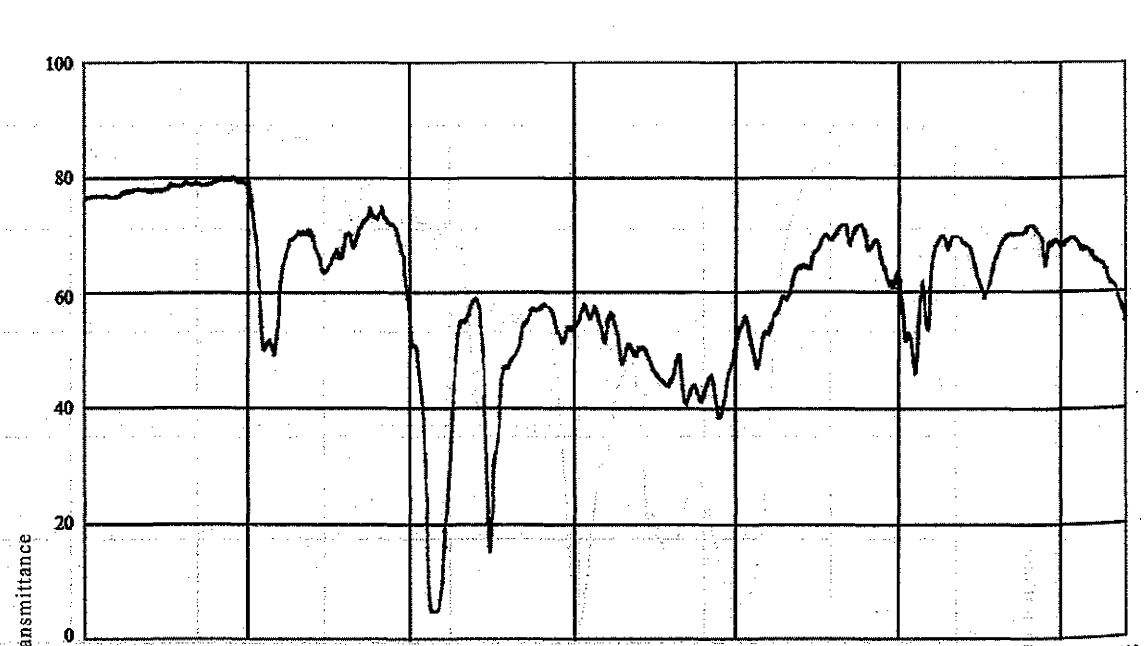
Phase: KBr disc



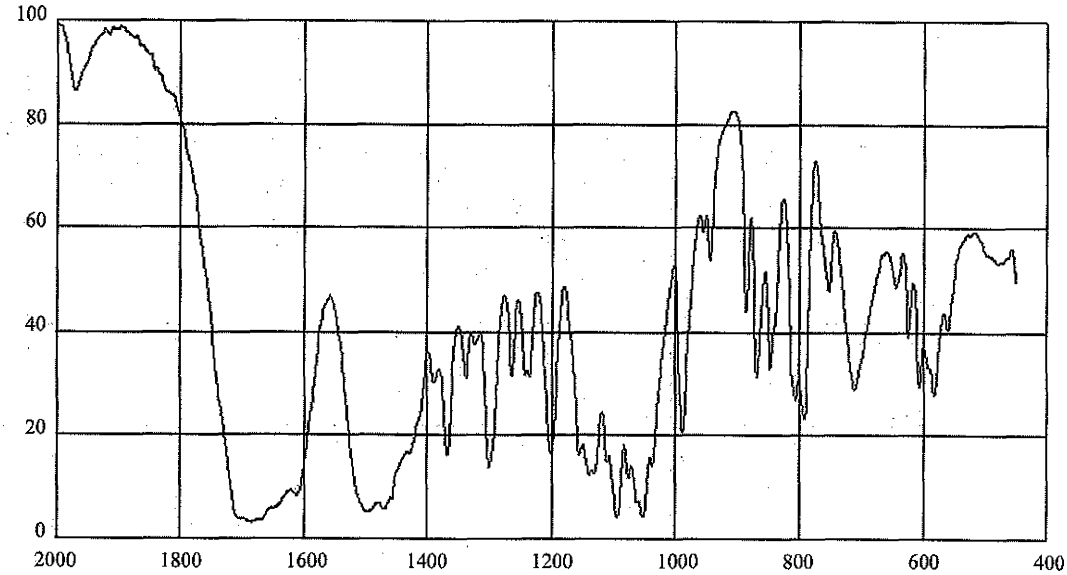
Atropine Sulphate

FTIR

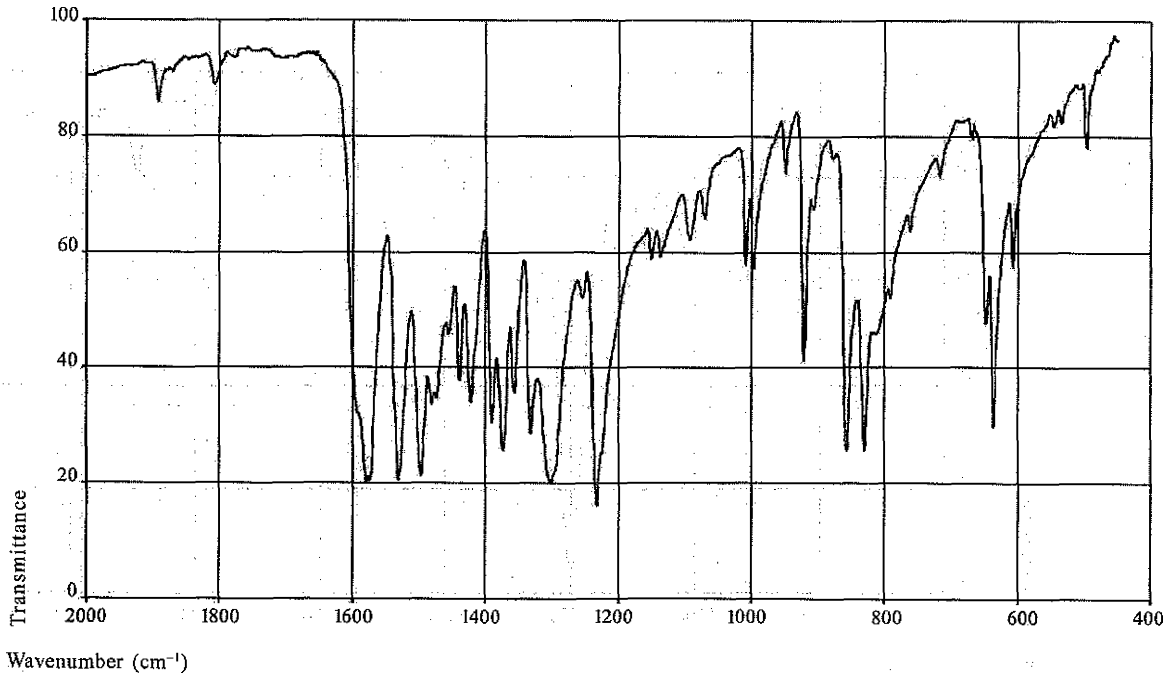
Phase: Liquid paraffin mull



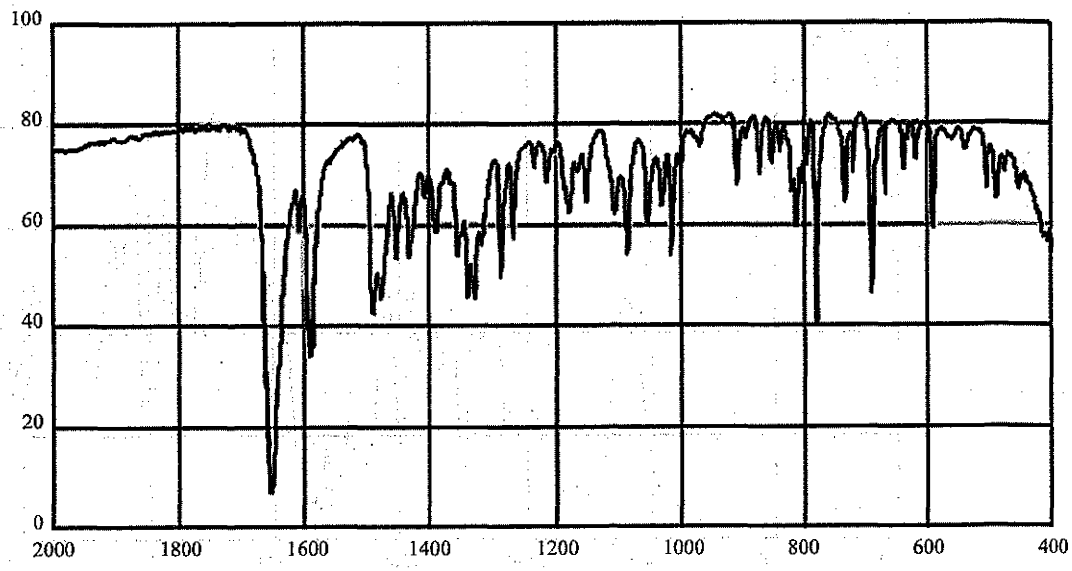
Azacitidine *FTIR* Phase: KBr disc



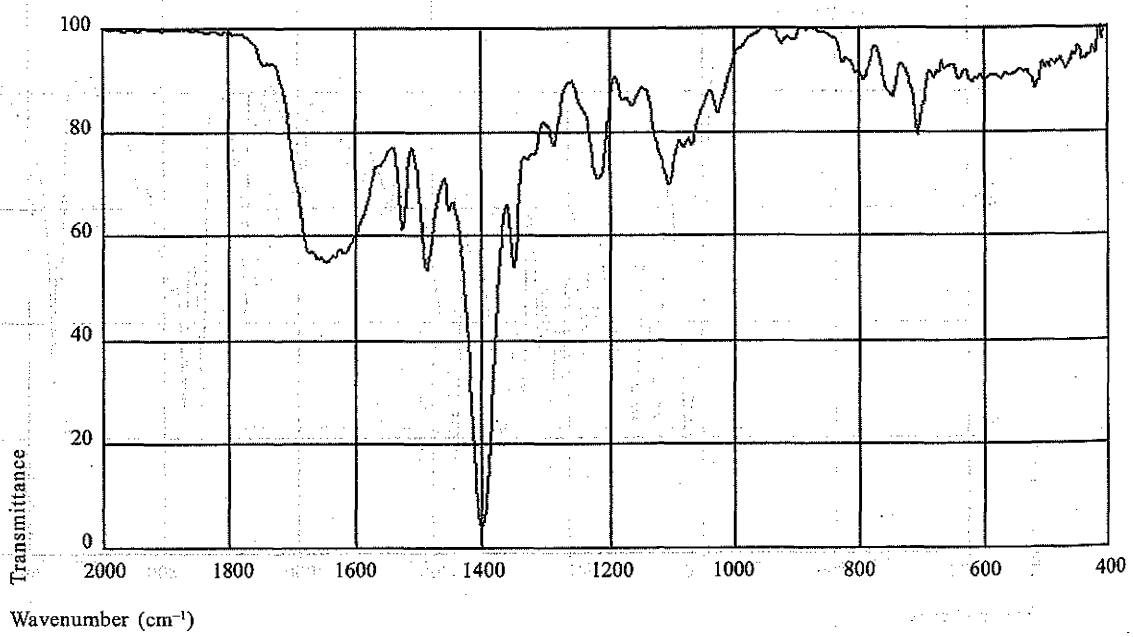
Azathioprine *FTIR* Phase: KBr disc



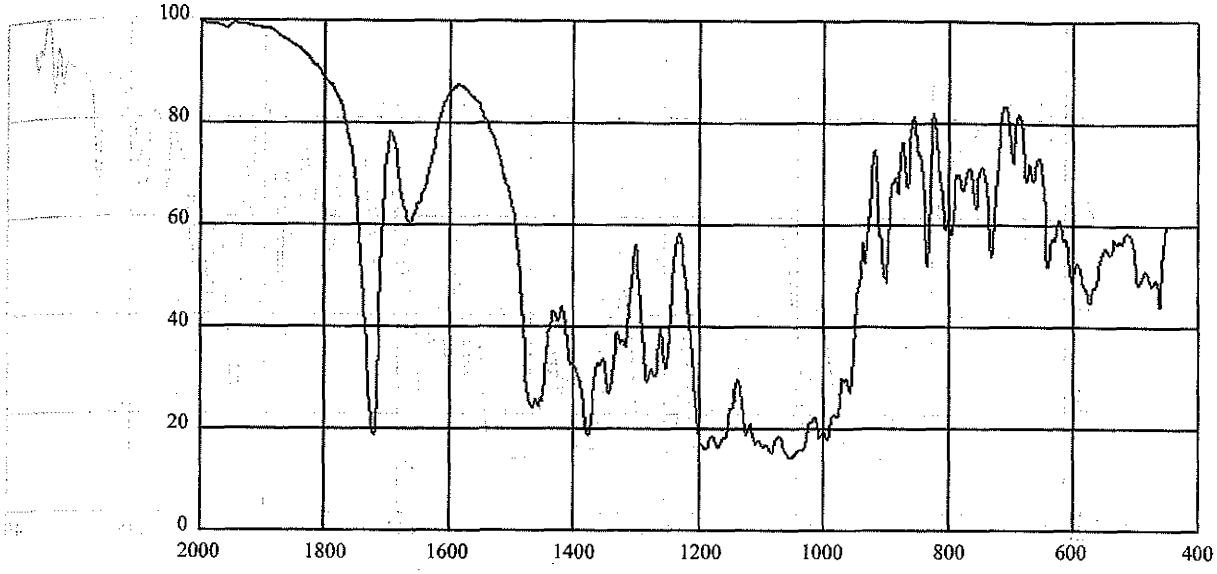
Azelastine Hydrochloride *FTIR* Phase: KBr disc



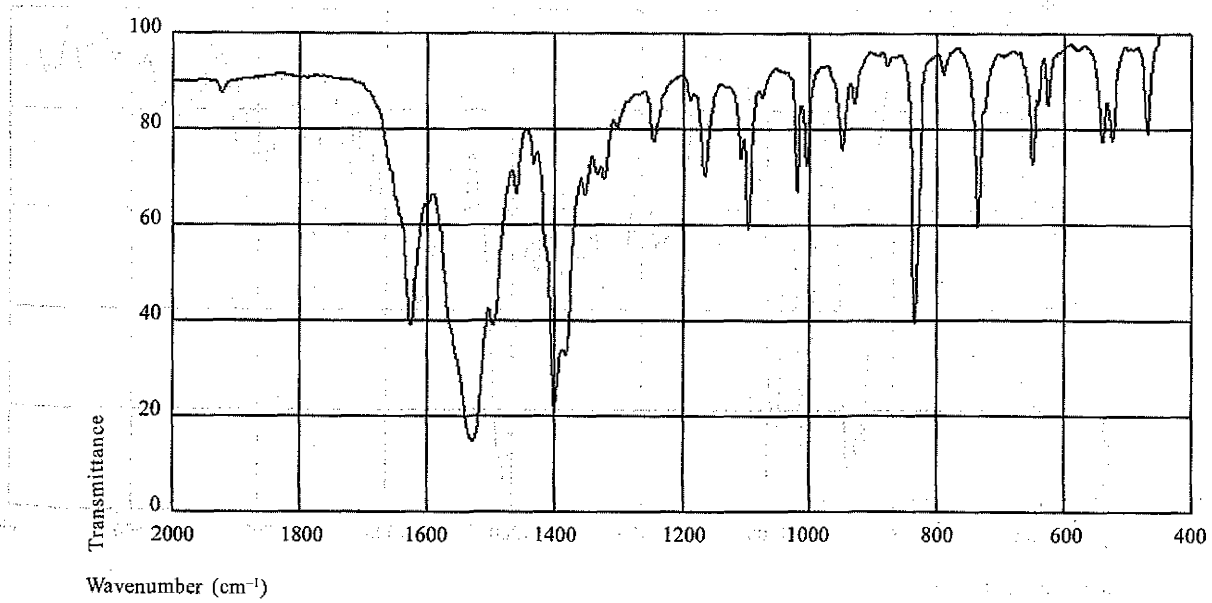
Azelnidipine *FTIR* Phase: KBr disc



Azithromycin *FTIR* Phase: KBr disc

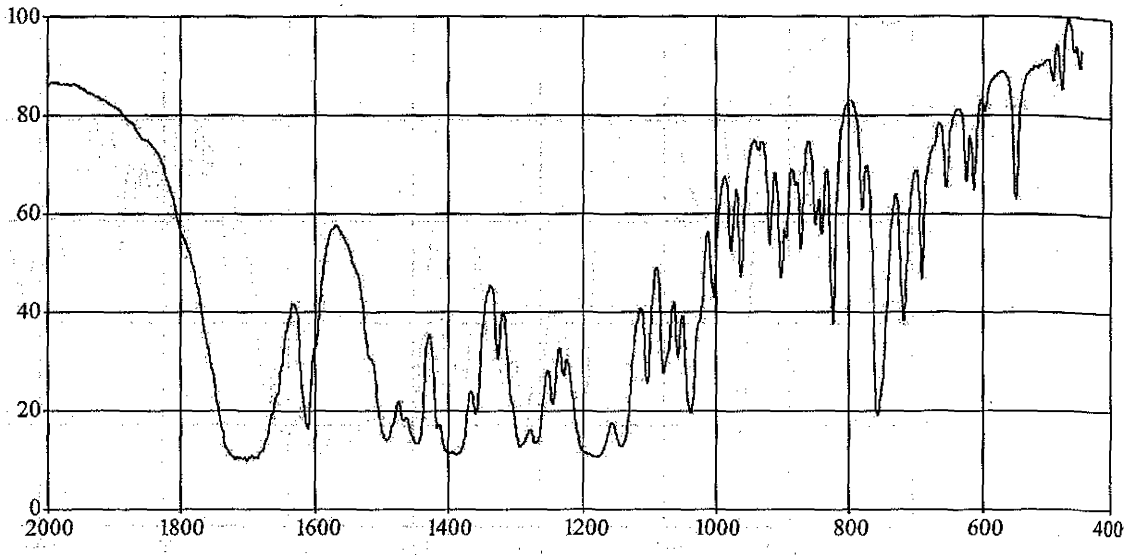


Baclofen *FTIR* Phase: KBr disc



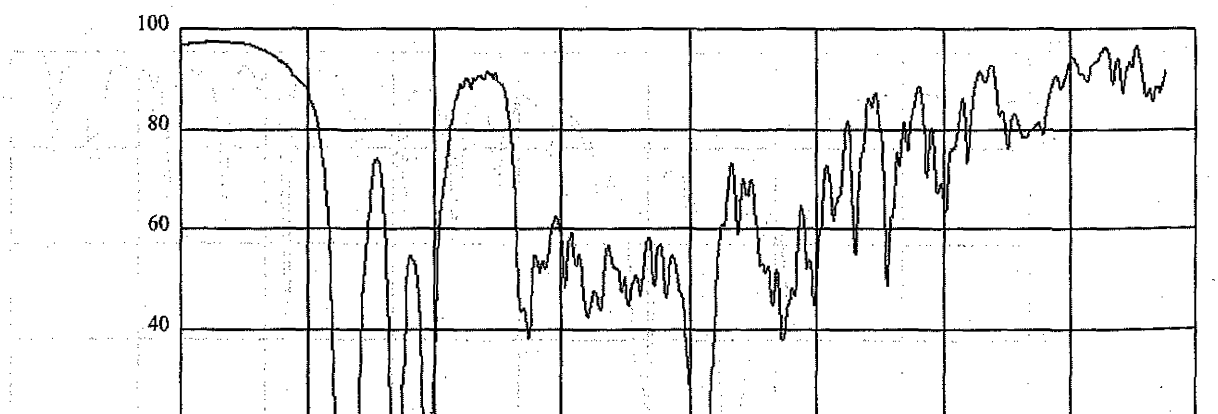
Bambuterol Hydrochloride

FTIR Phase: KBr disc



Beclomethasone Dipropionate

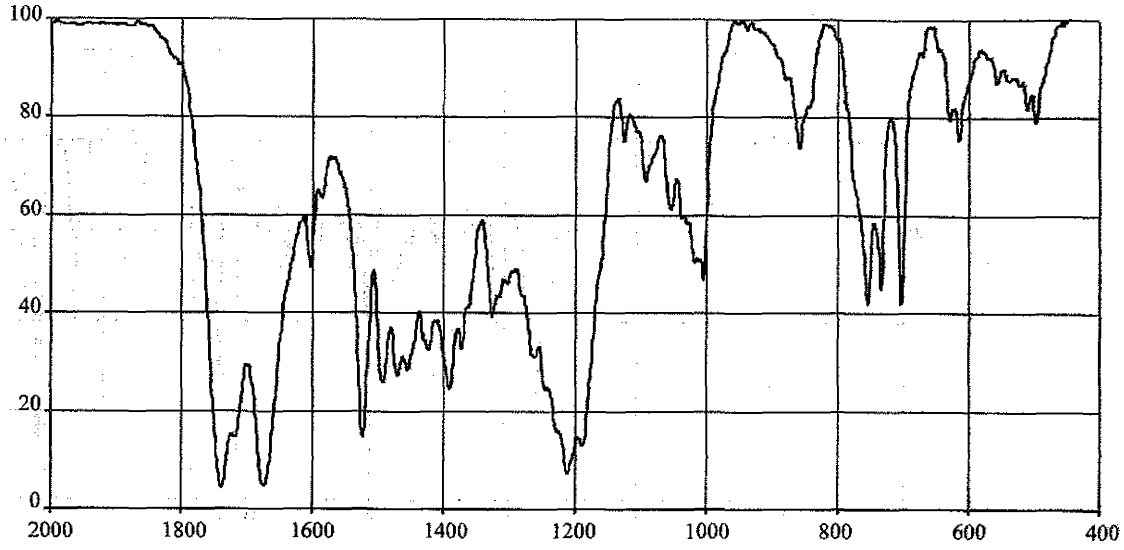
FTIR Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



Benazepril Hydrochloride

FTIR

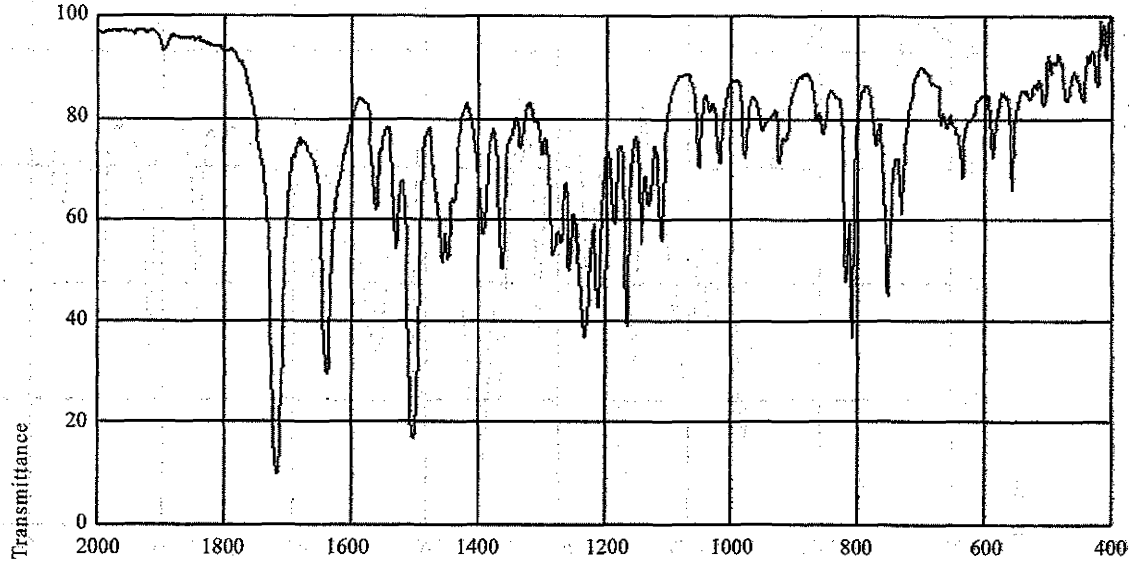
Phase: KBr disc



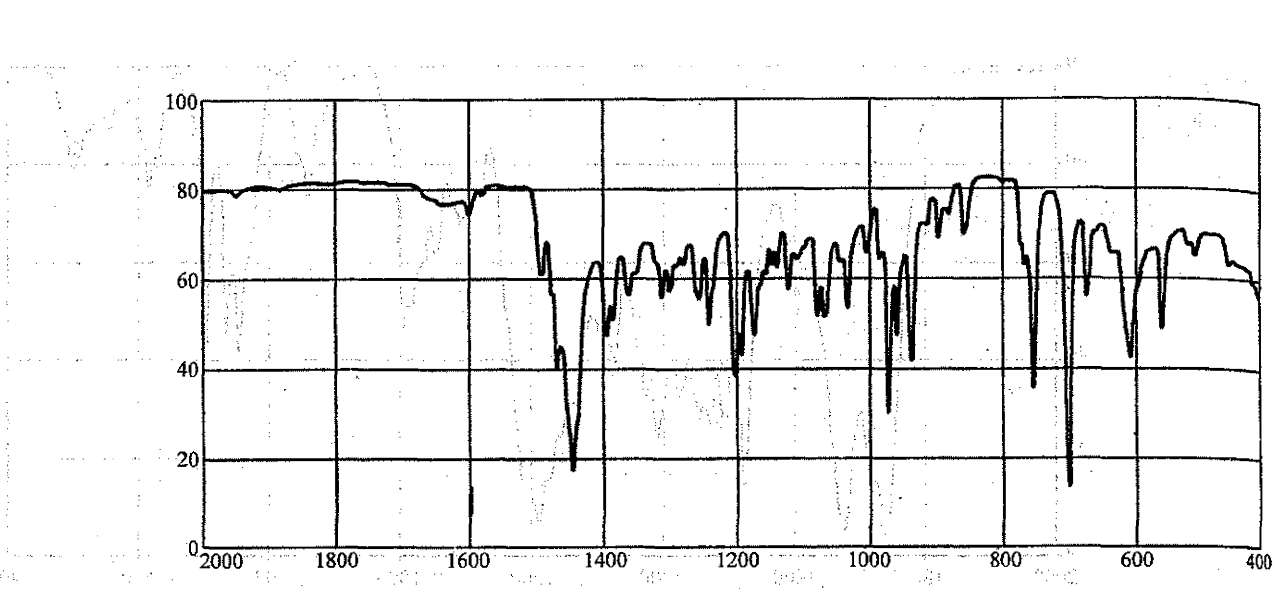
Bendamustine Hydrochloride

FTIR

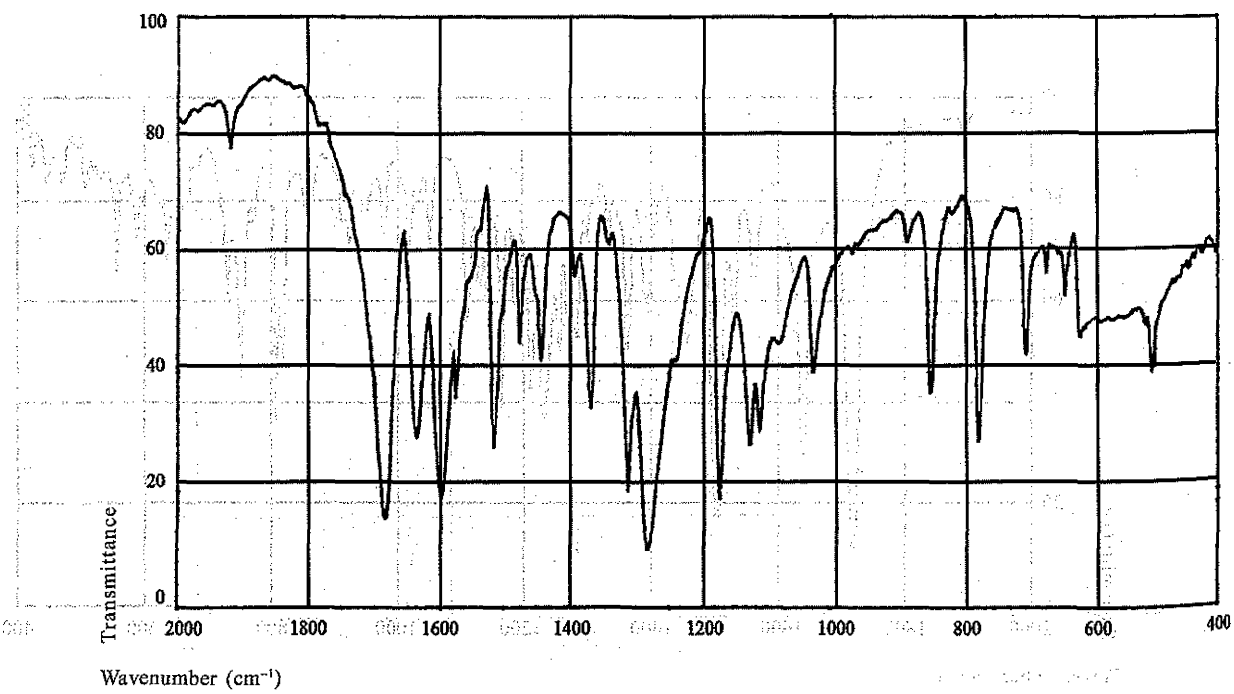
Phase: KBr disc



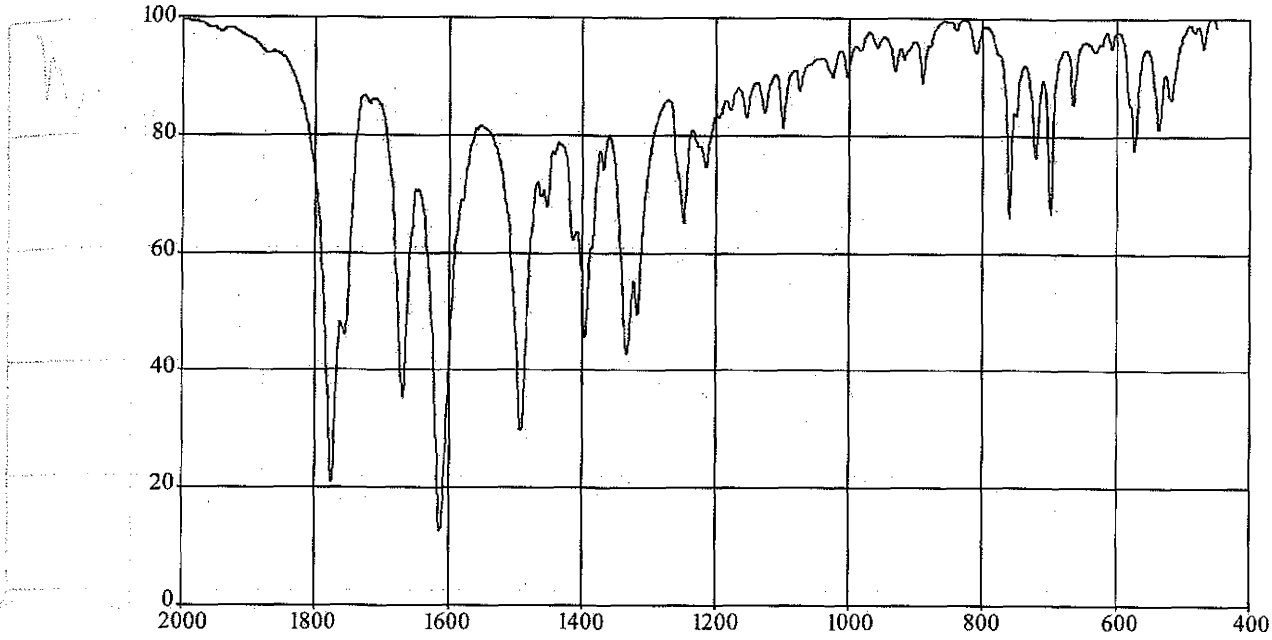
Benzhexol Hydrochloride *Dispersive IR* Phase: KCl disc



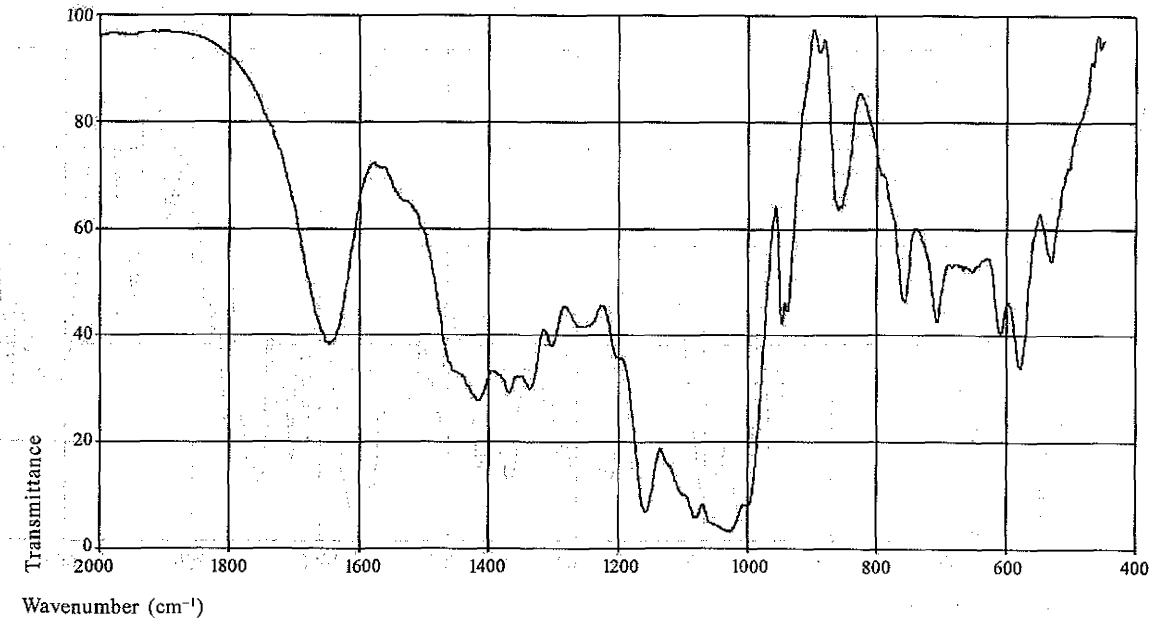
Benzocaine *FTIR* Phase: KBr disc



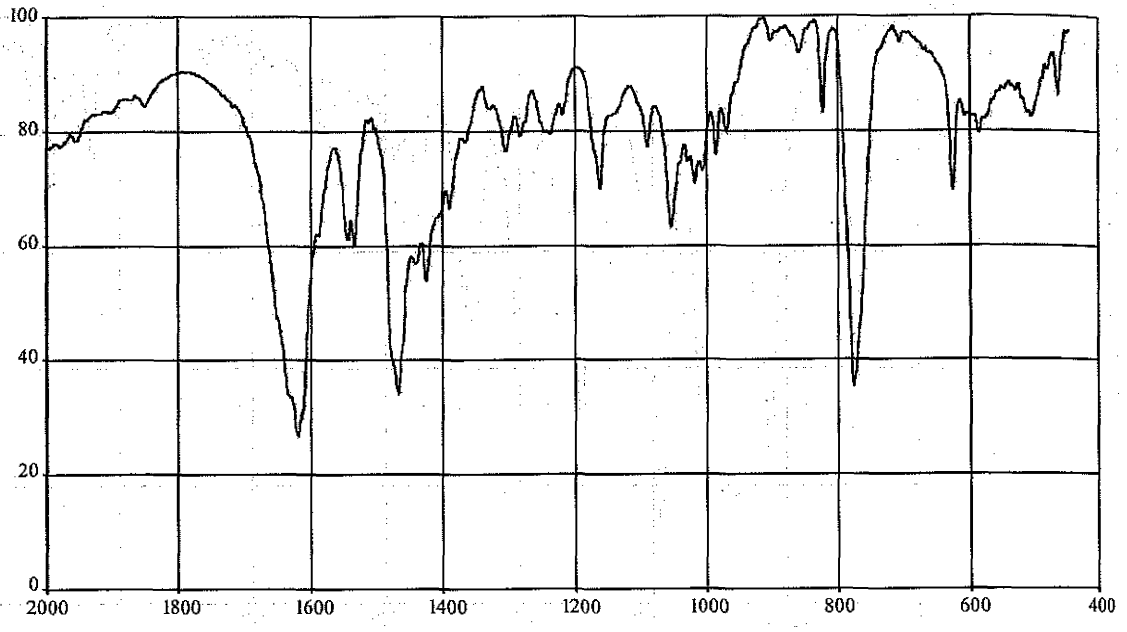
Benzylpenicillin Potassium *FTIR* Phase: KBr disc



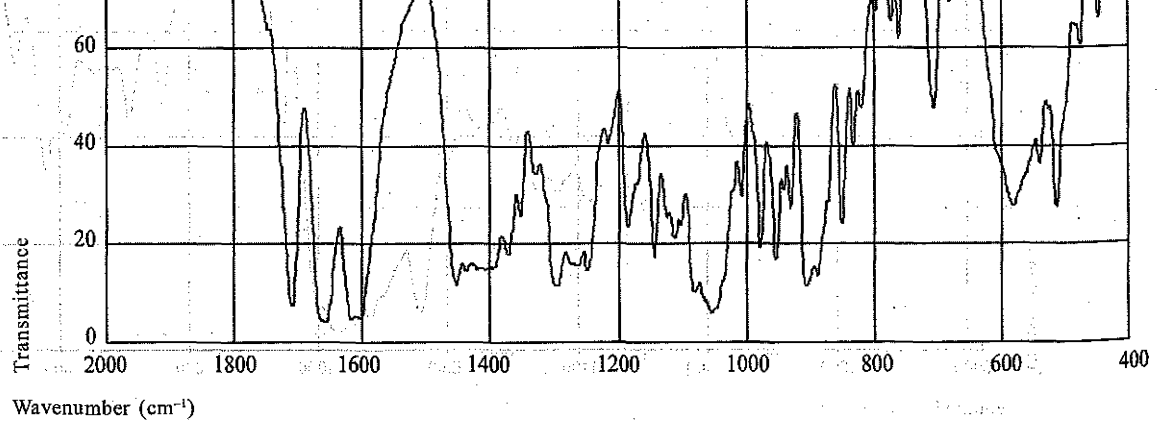
Betacyclodextrin *FTIR* Phase: KBr disc



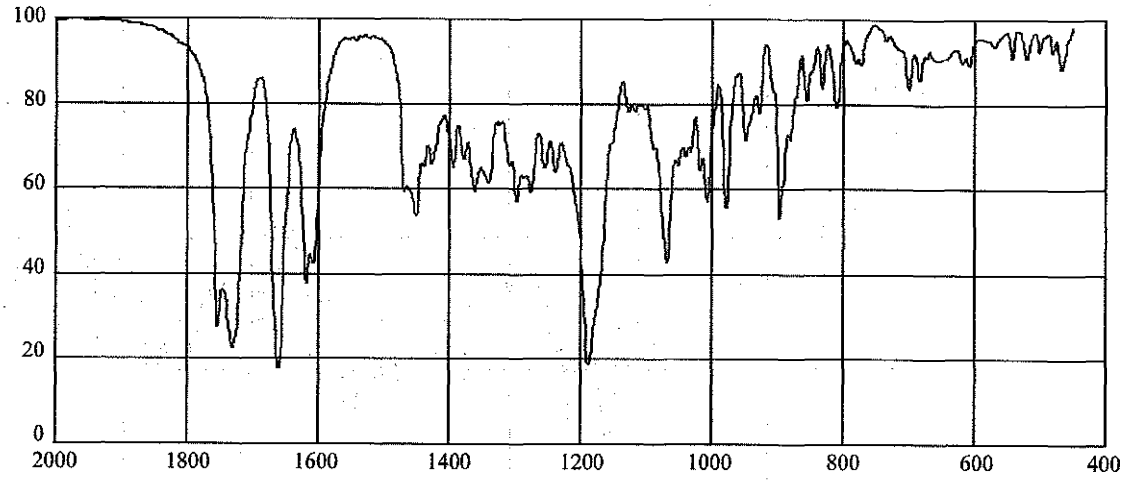
Betahistine Hydrochloride *FTIR* Phase: KBr disc



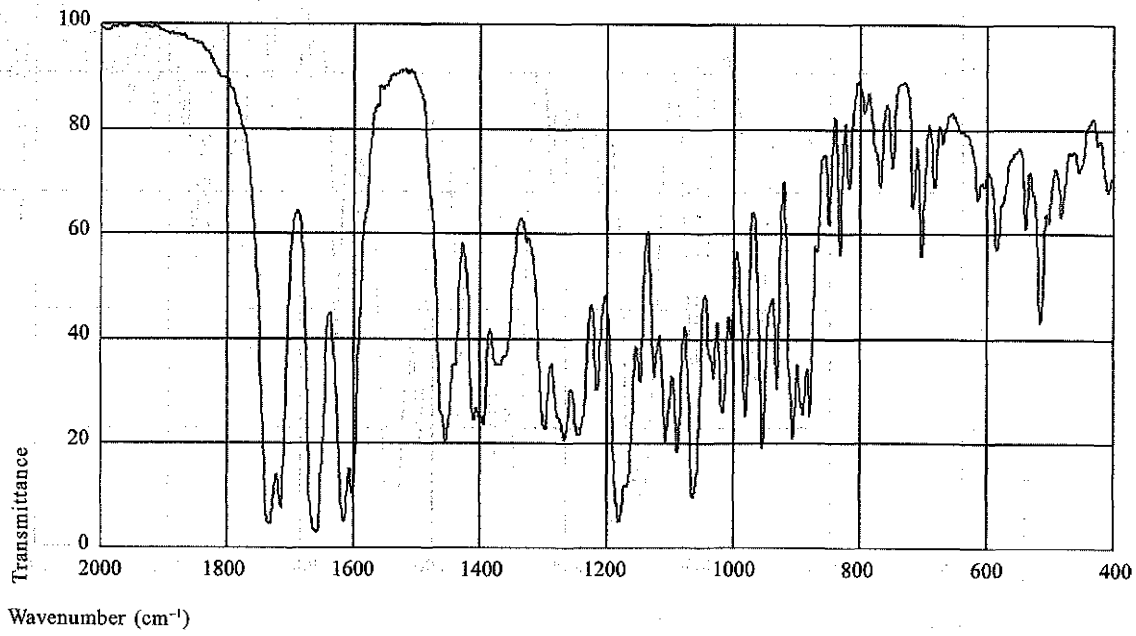
Betamethasone *FTIR* Phase: KBr disc



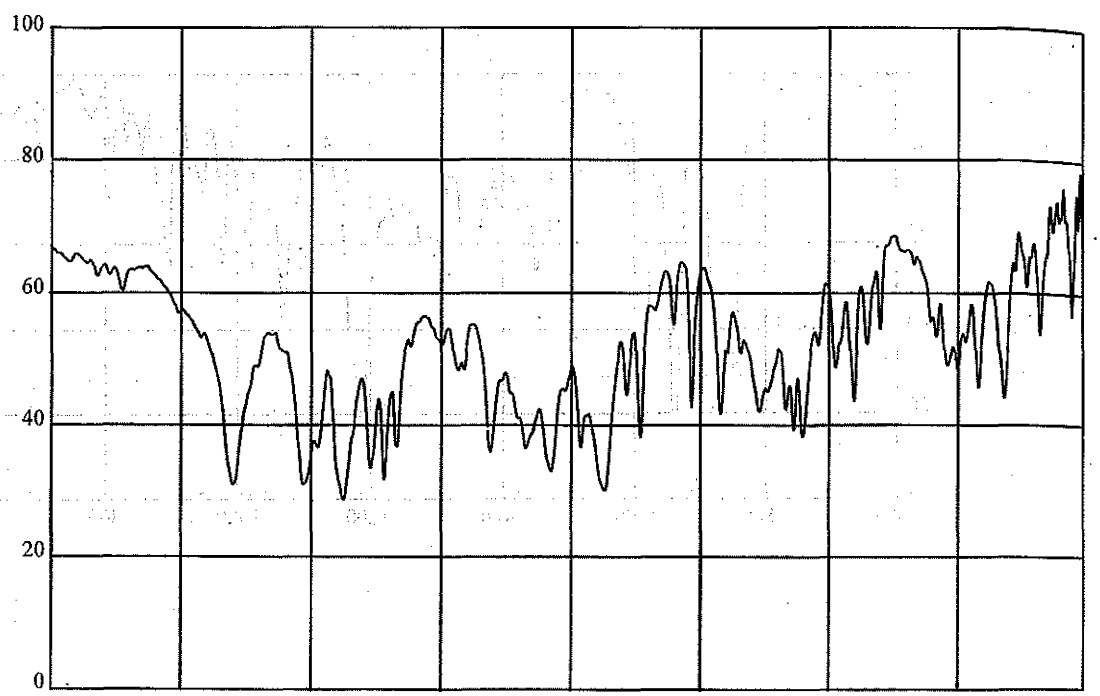
Betamethasone Dipropionate *FTIR* Phase: KBr disc



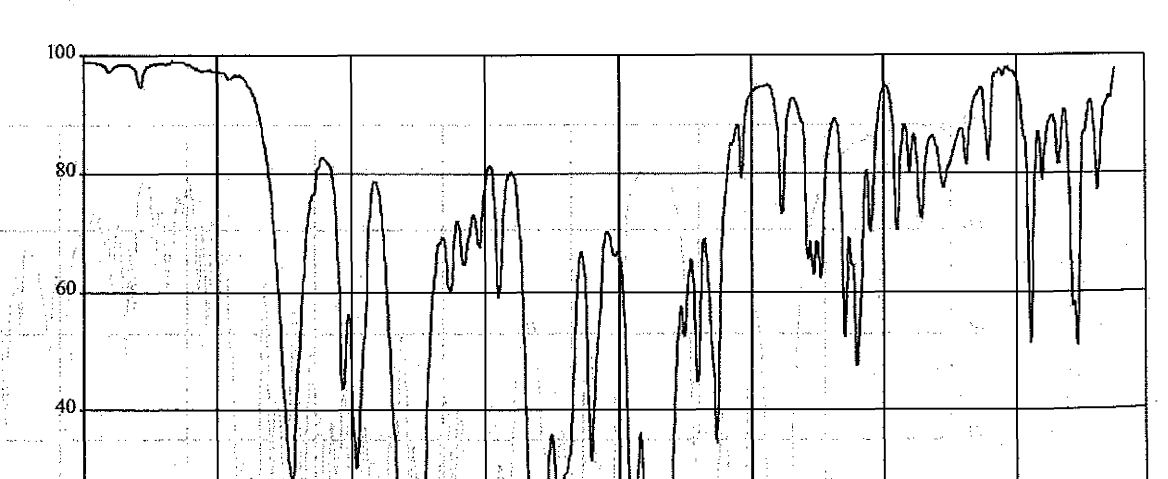
Betamethasone Valerate *FTIR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



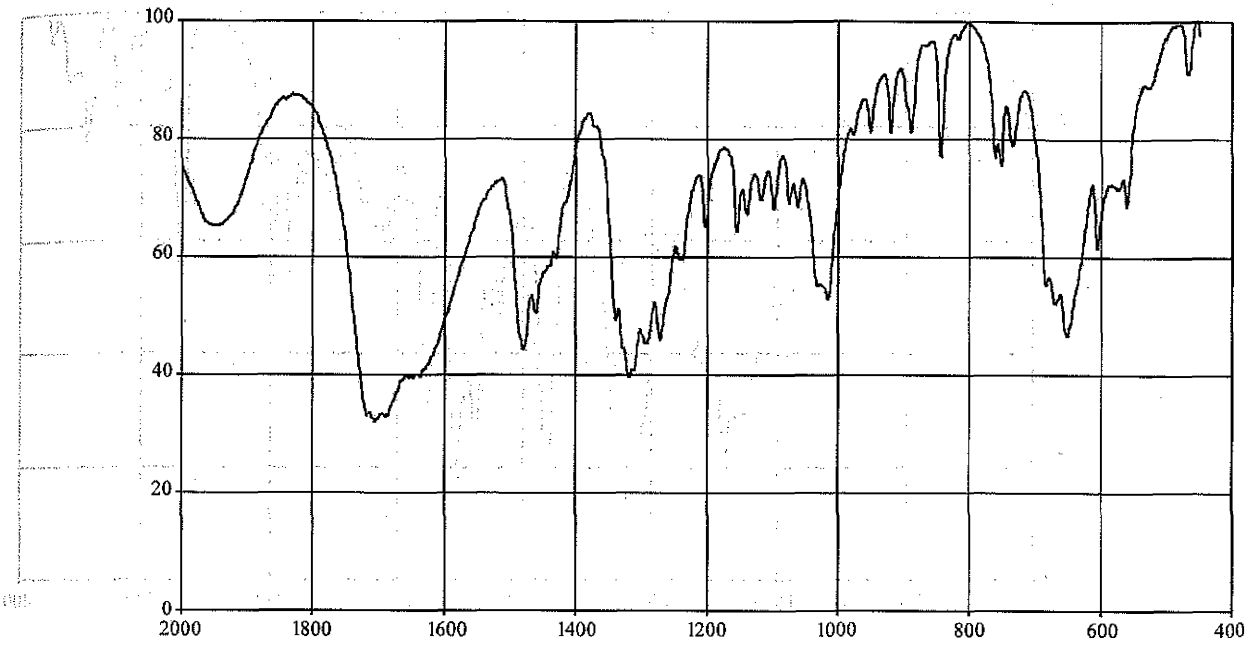
Bezafibrate *FTIR* Phase: KBr disc



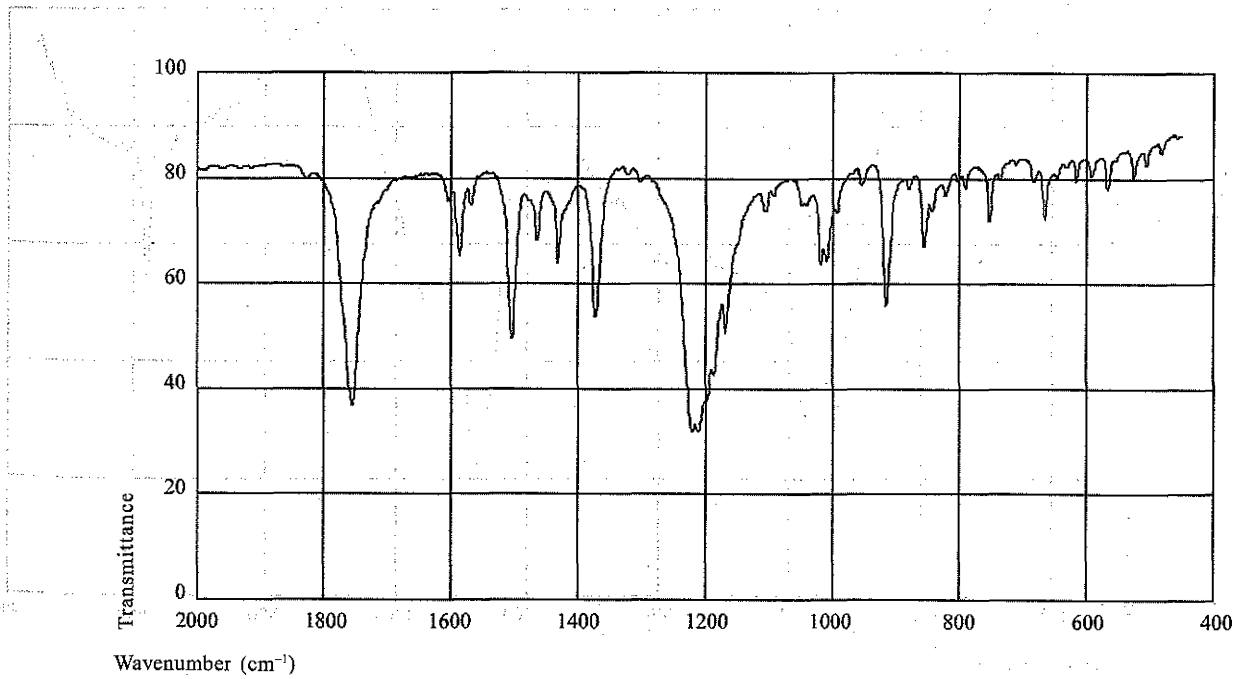
Bicalutamide *FTIR* Phase: KBr disc



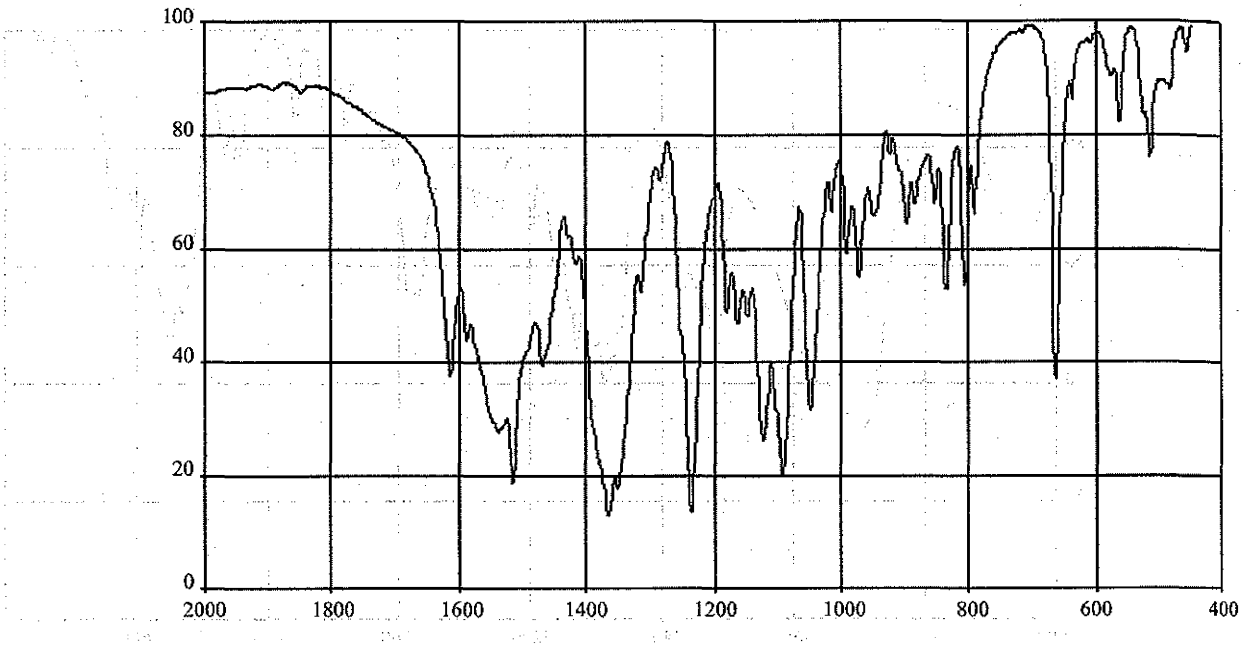
Biotin *FTIR* Phase: KBr disc



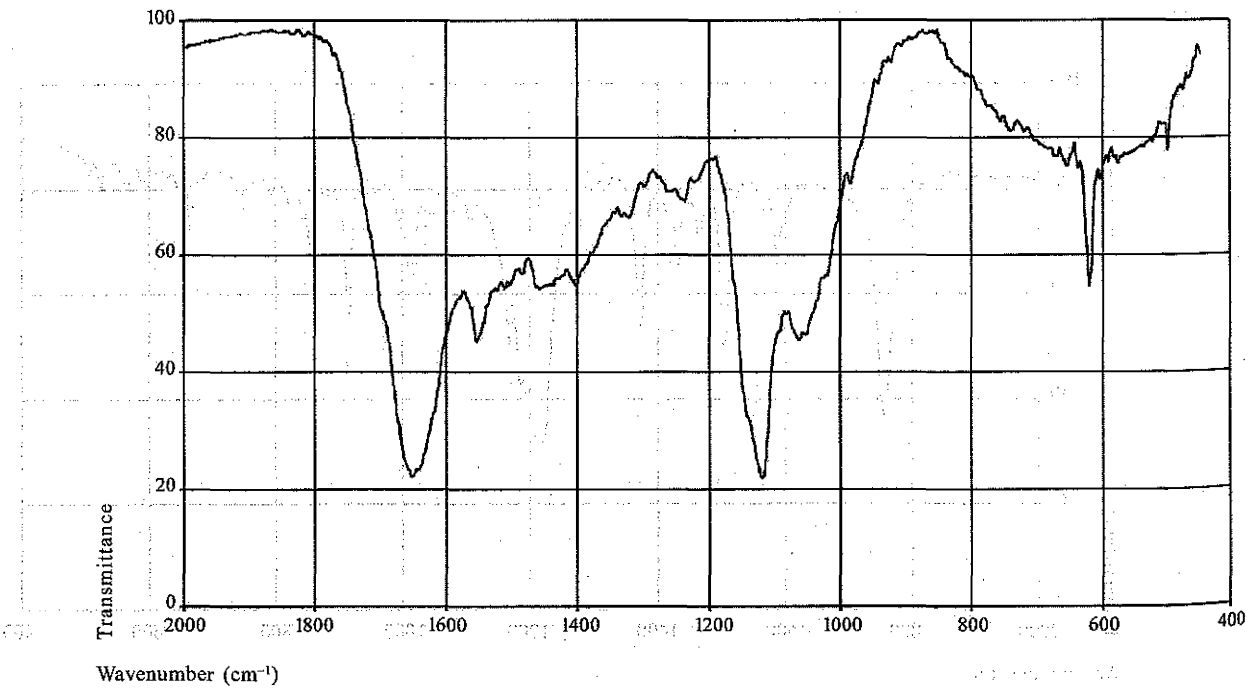
Bisacodyl *FTIR* Phase: KBr disc



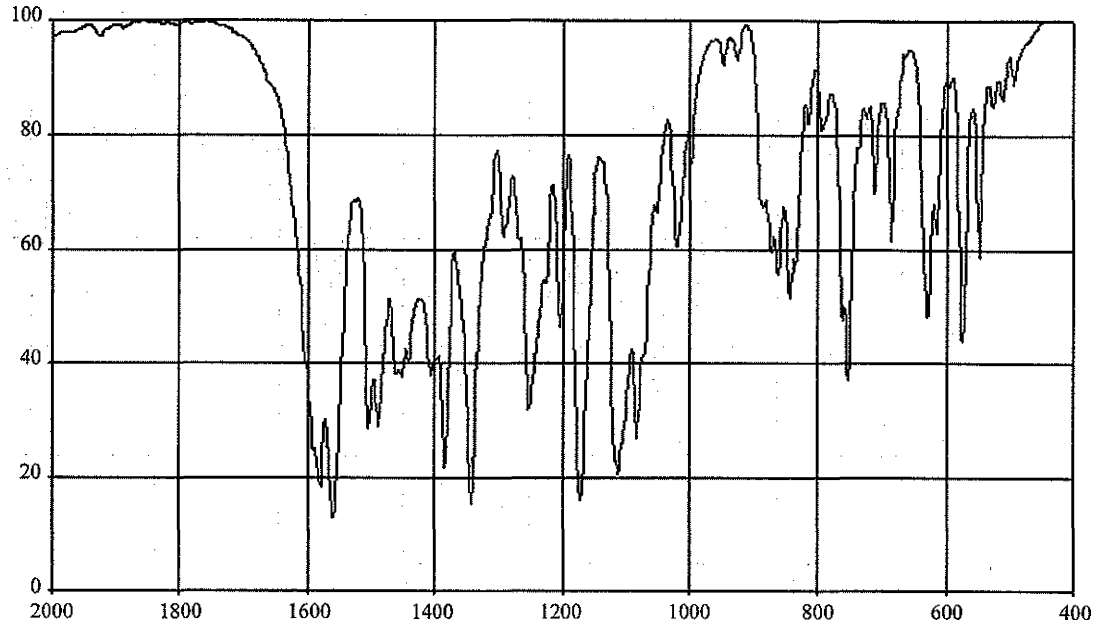
Bisoprolol Fumarate *FTIR* Phase: KBr disc



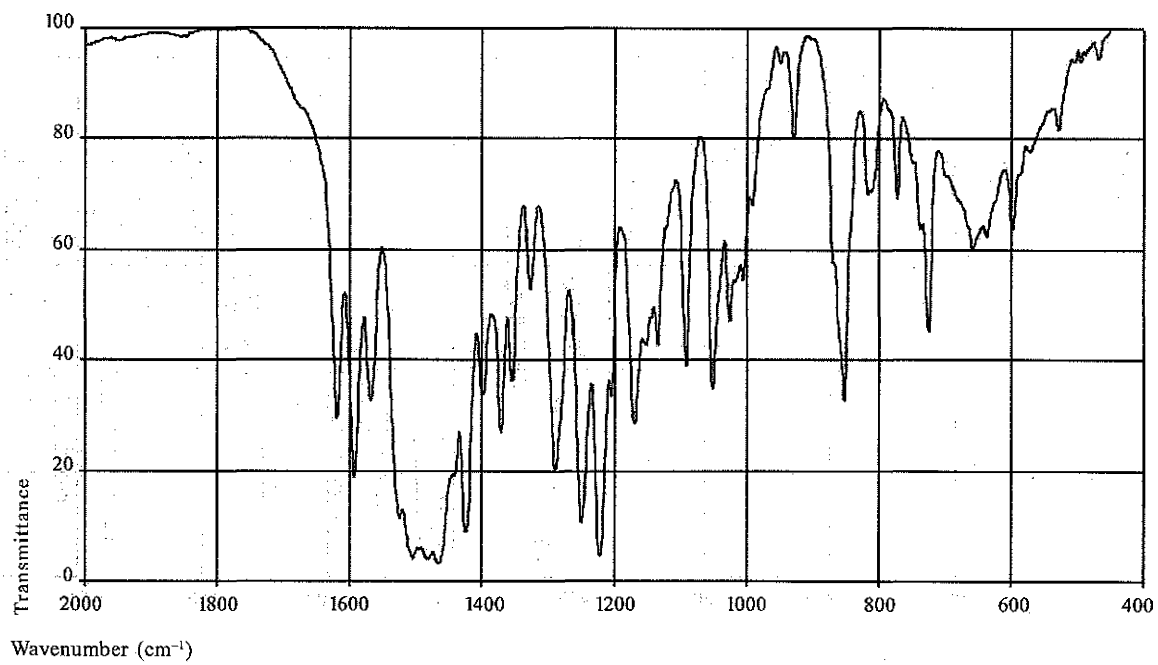
Bleomycin Sulphate *FTIR* Phase: KBr disc



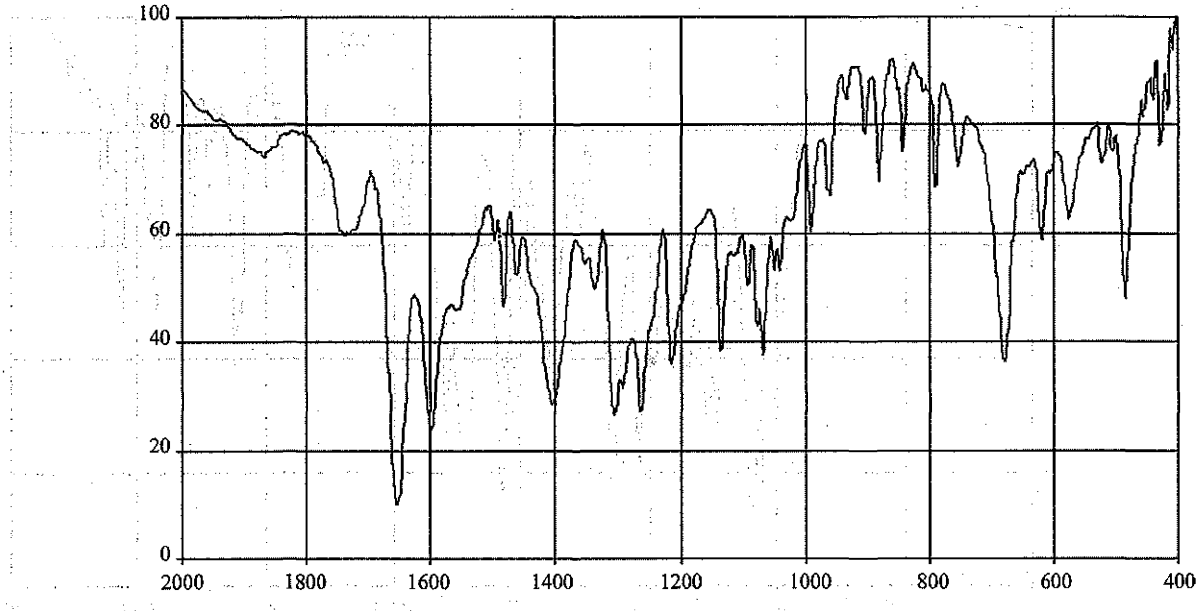
Bosentan Monohydrate *FTIR* Phase: KBr disc



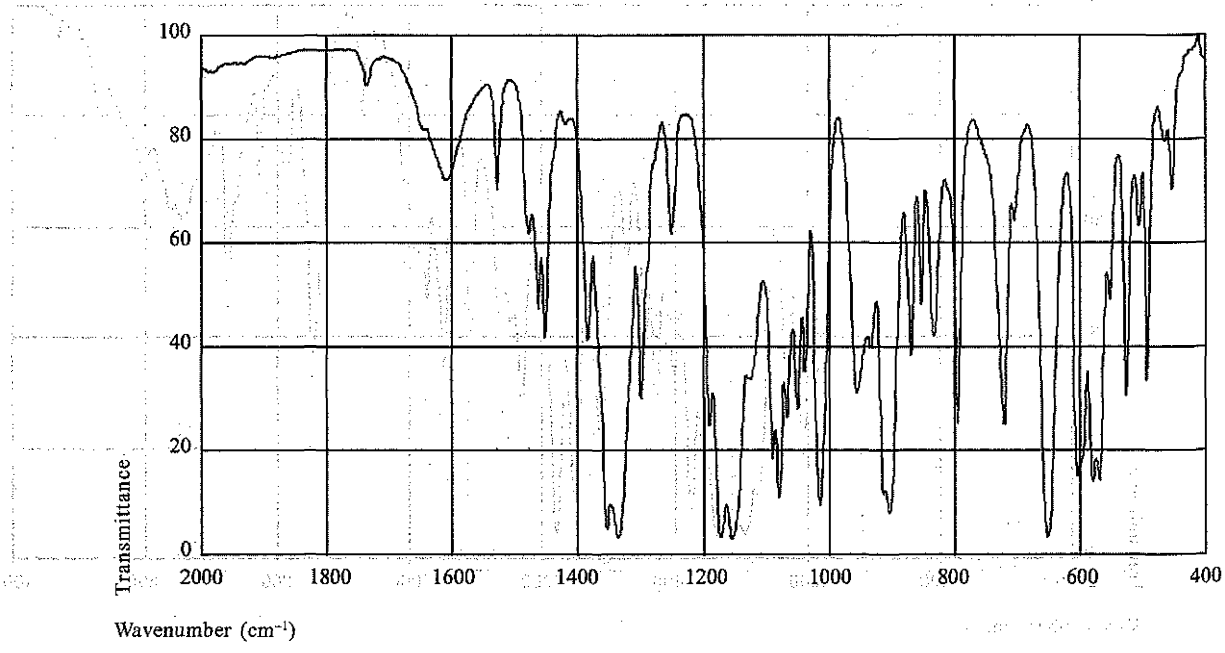
Bosutinib *FTIR* Phase: KBr disc



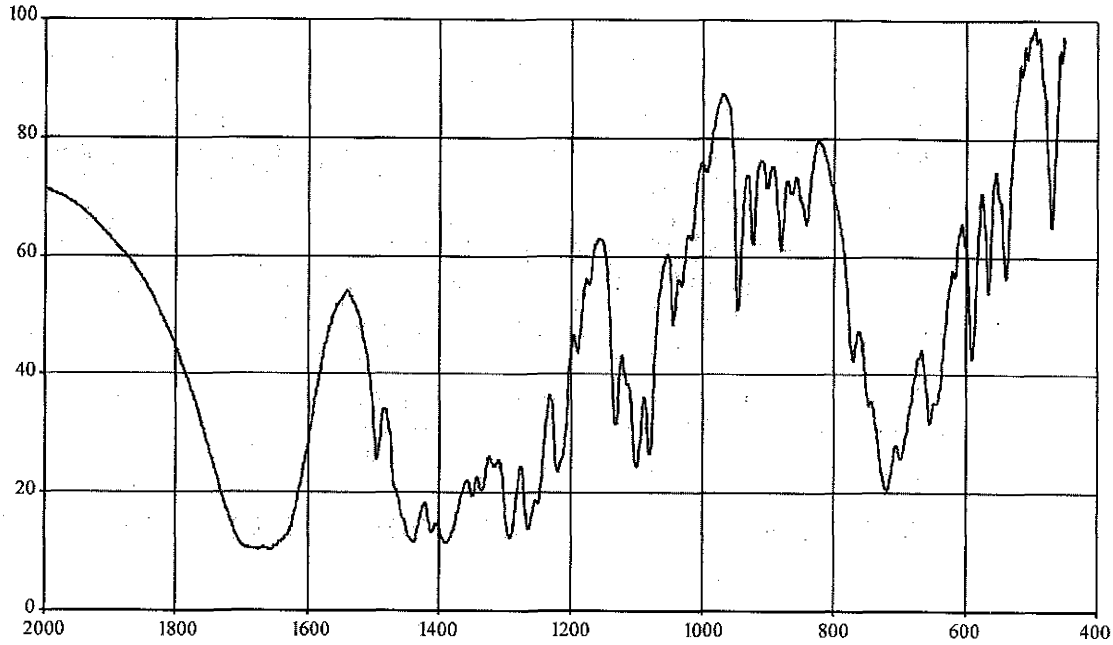
Brimonidine Tartrate *FTIR* Phase: KBr disc



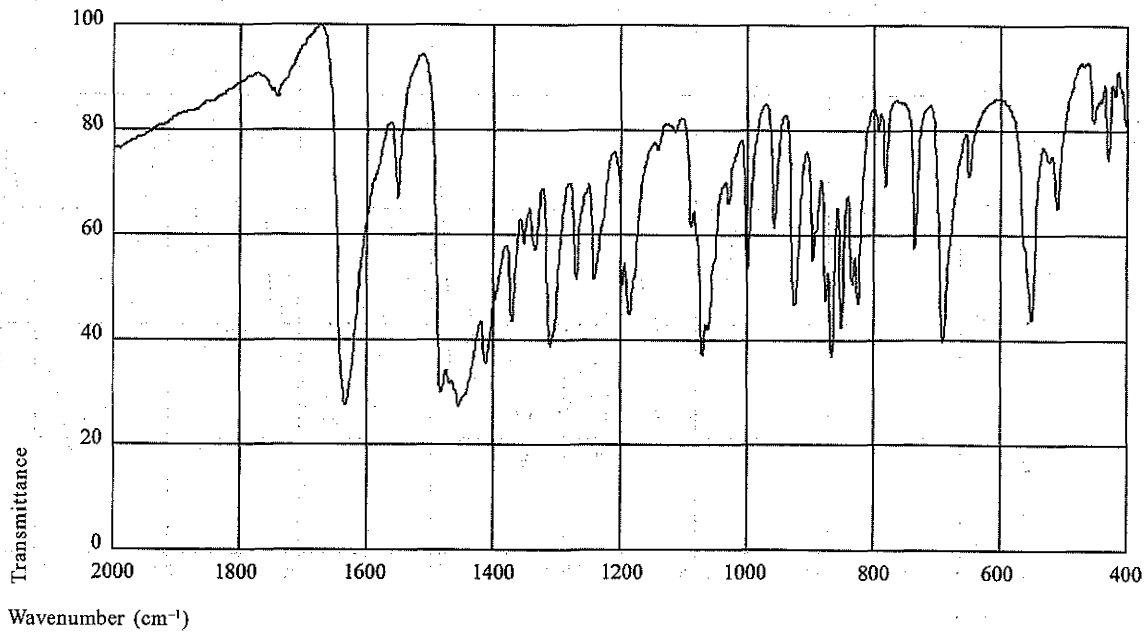
Brinzolamide *FTIR* Phase: KBr disc



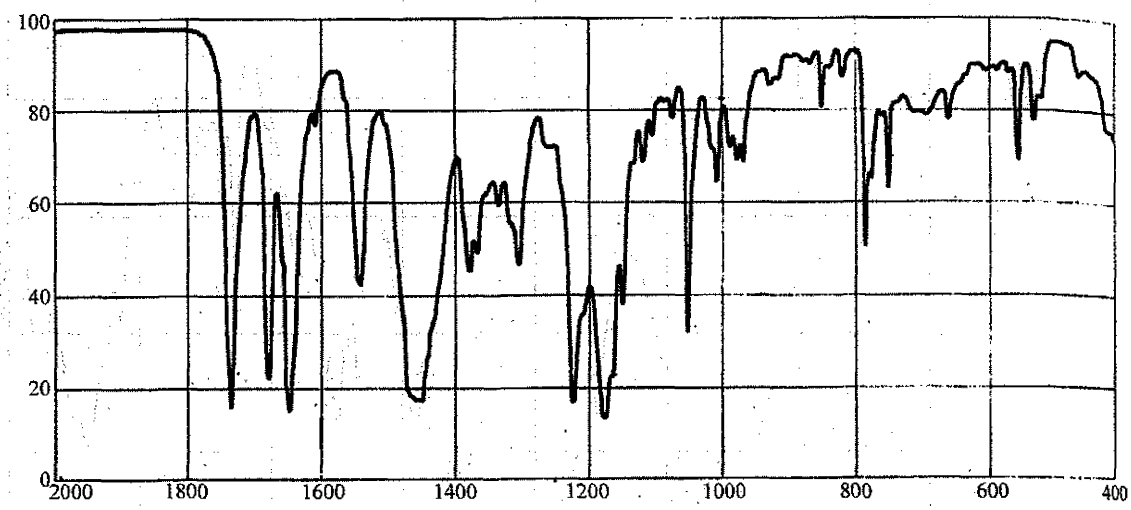
Brivaracetam *FTIR* Phase: KBr disc



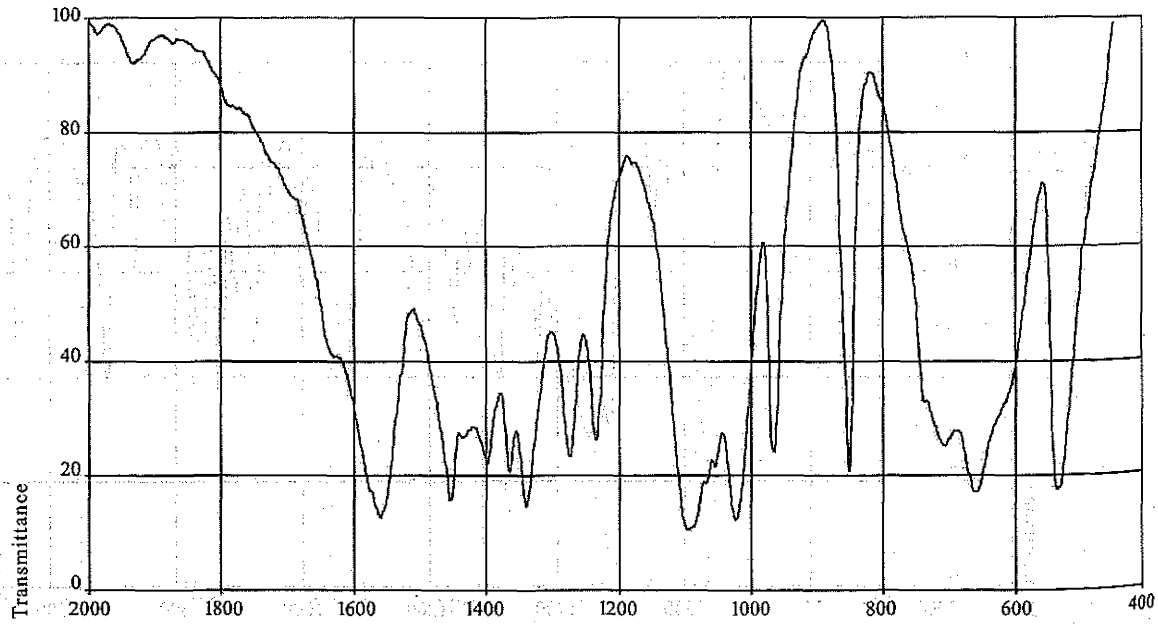
Bromhexine Hydrochloride *FTIR* Phase: KCl disc



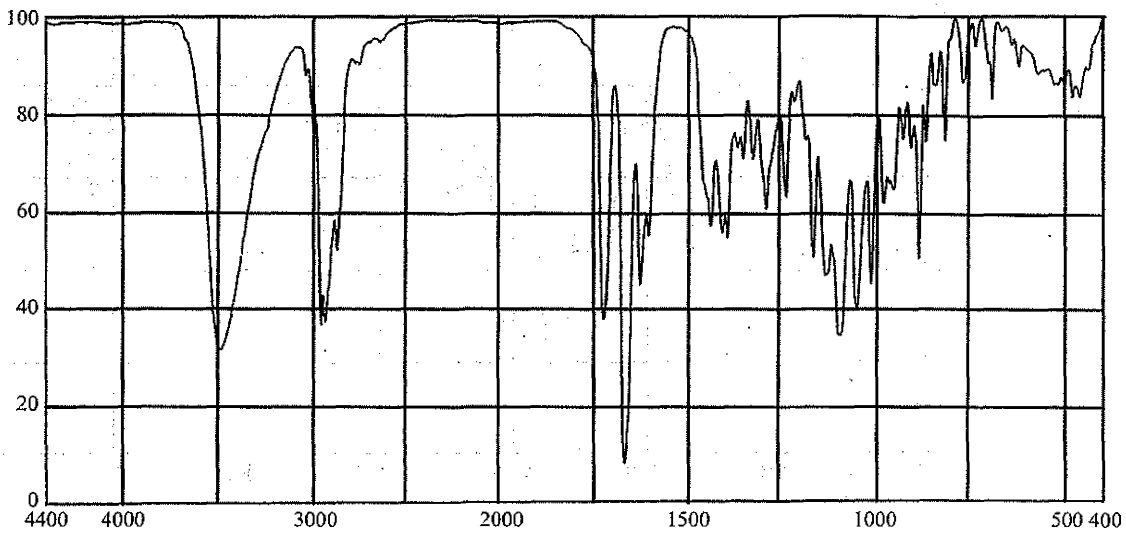
Bromocriptine Mesylate *Dispersive IR* Phase: Liquid paraffin mull



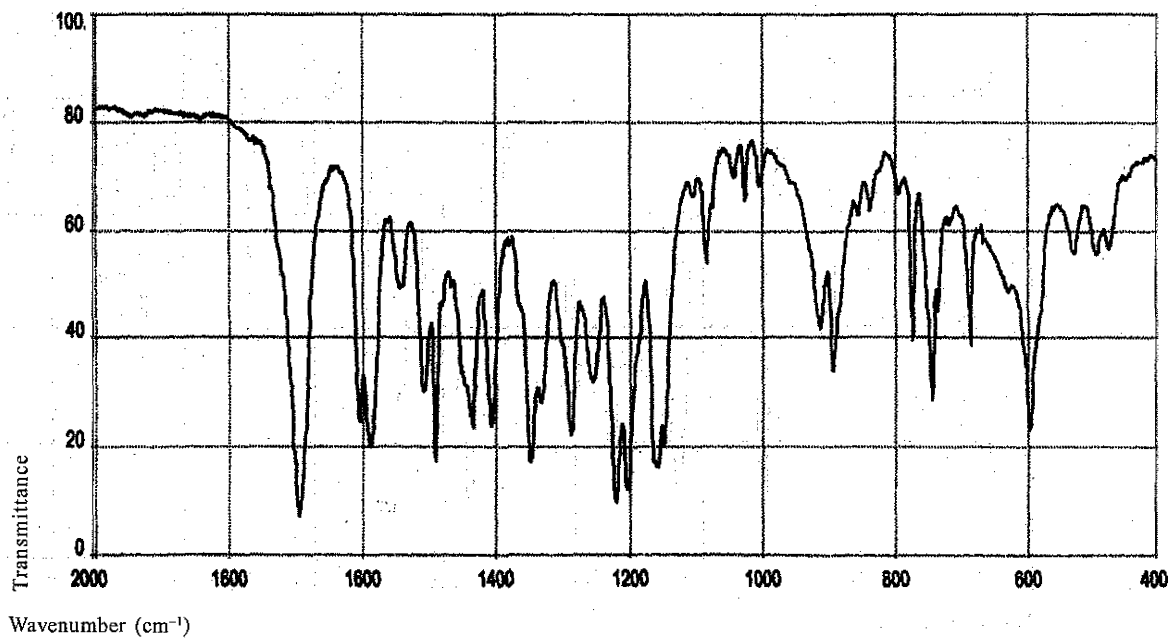
Bronopol *FTIR* Phase: KBr disc



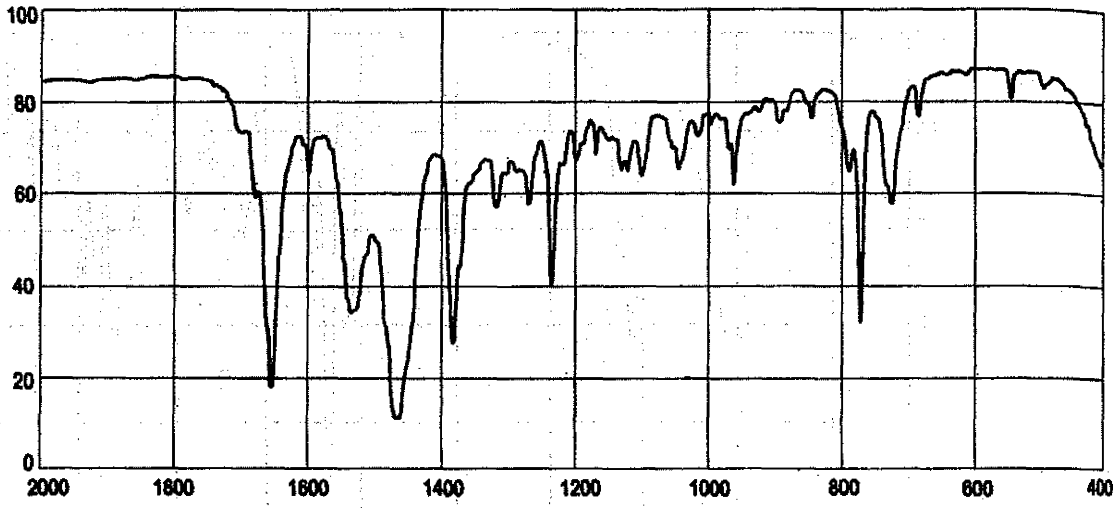
Budesonide *FTIR* Phase: KBr disc



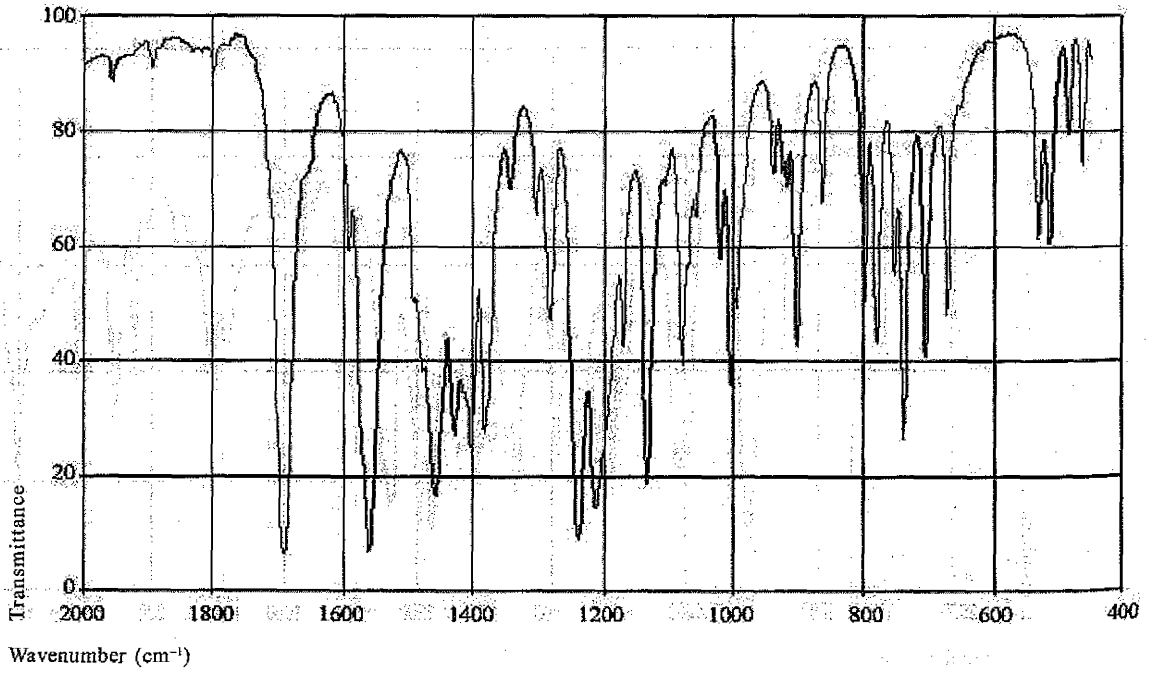
Bumetanide *FTIR* Phase: KBr disc



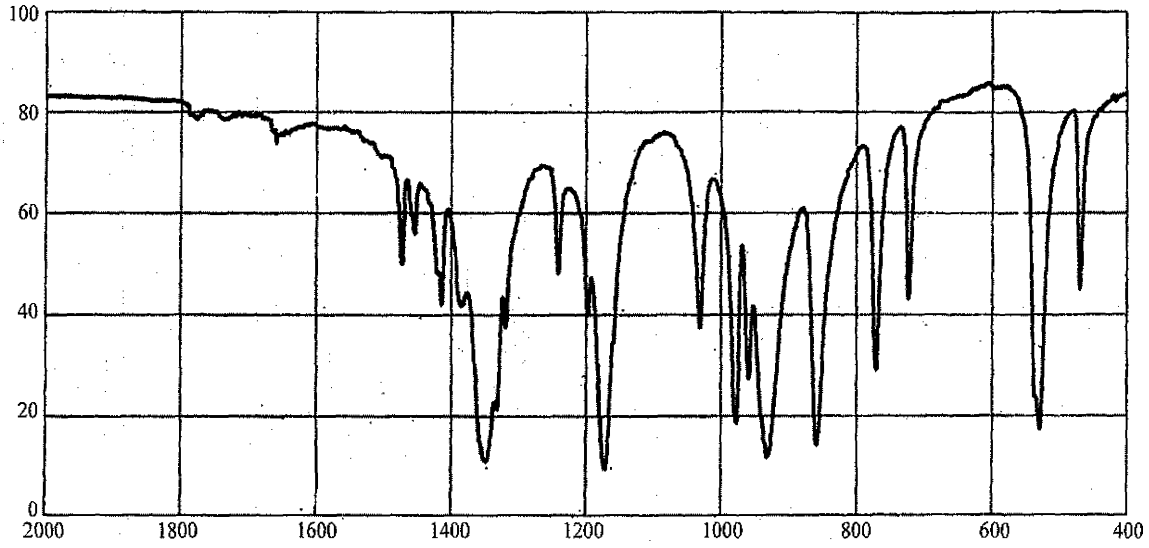
Bupivacaine Hydrochloride *Dispersive IR* Phase: Liquid paraffin mull



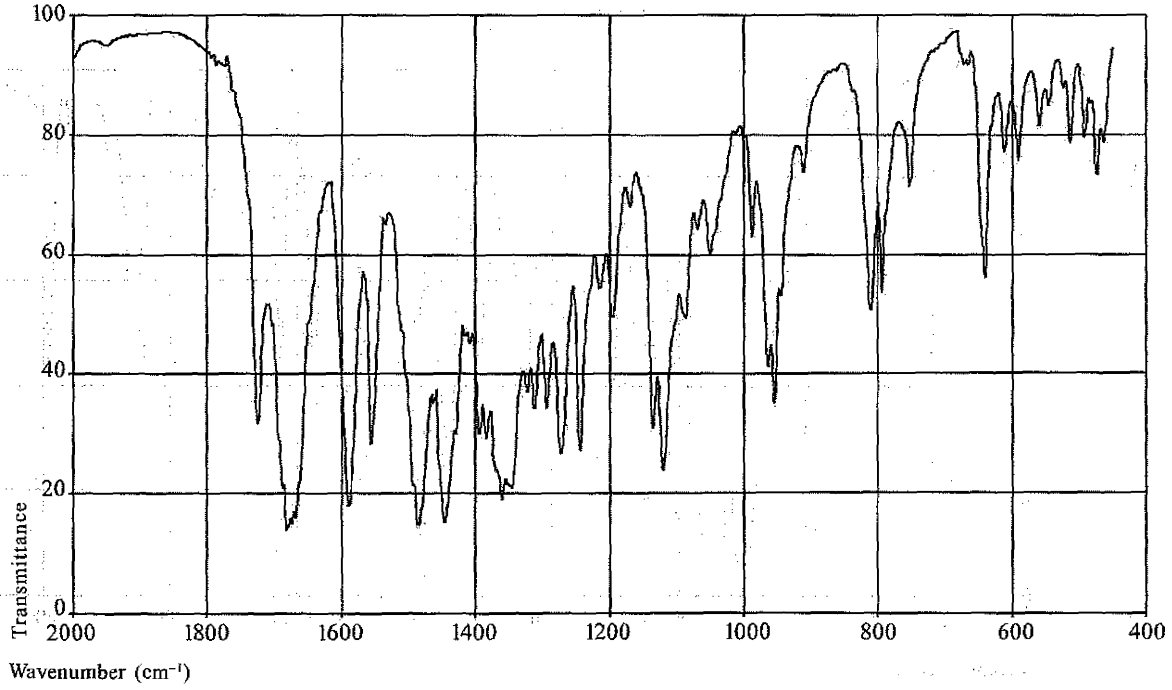
Bupropion Hydrochloride *FTIR* Phase: KBr disc



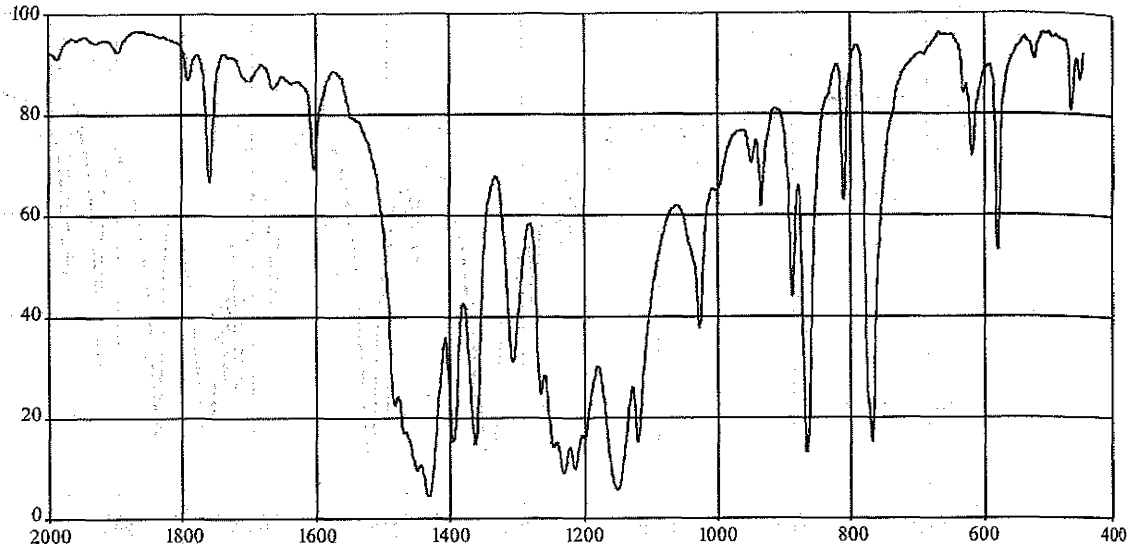
Busulphan *Dispersive IR* Phase: KBr disc



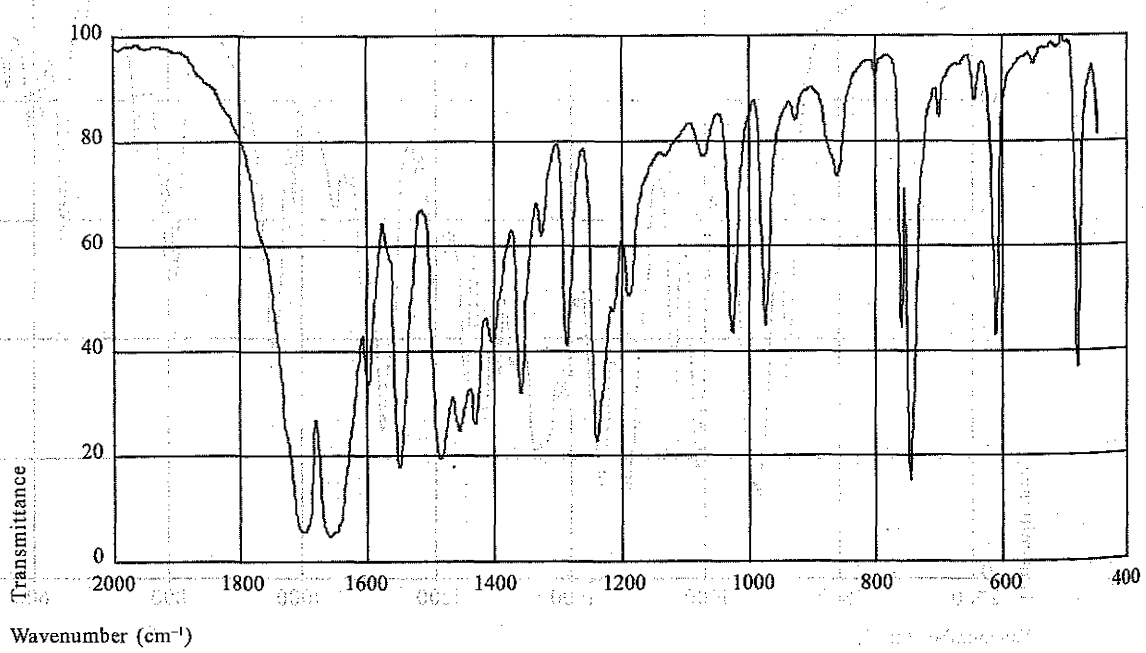
Buspirone Hydrochloride *Dispersive IR* Phase: KBr disc



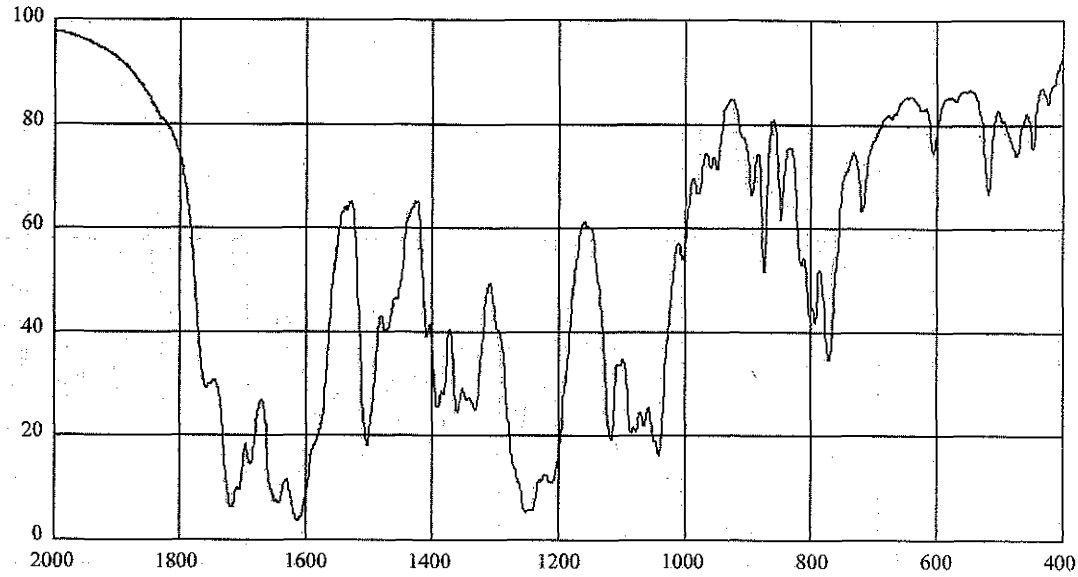
Butylated Hydroxytoluene *Dispersive IR* Phase: KBr disc



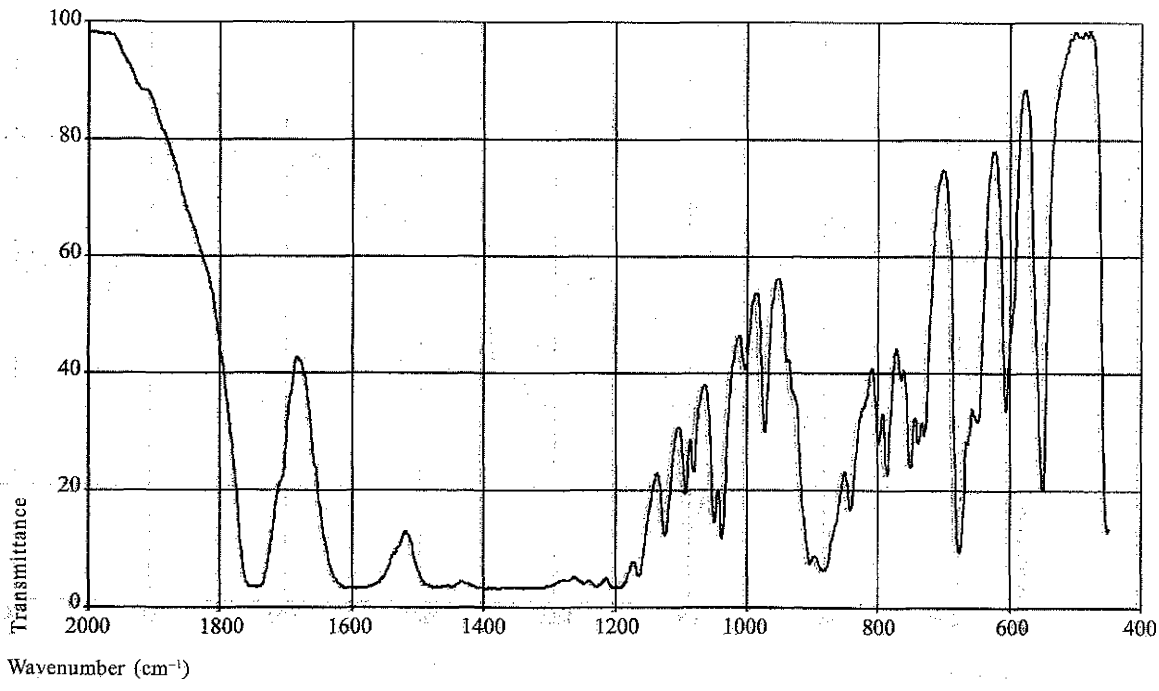
Caffeine *FTIR* Phase: KBr disc



Capecitabine *FTIR* Phase: KBr disc



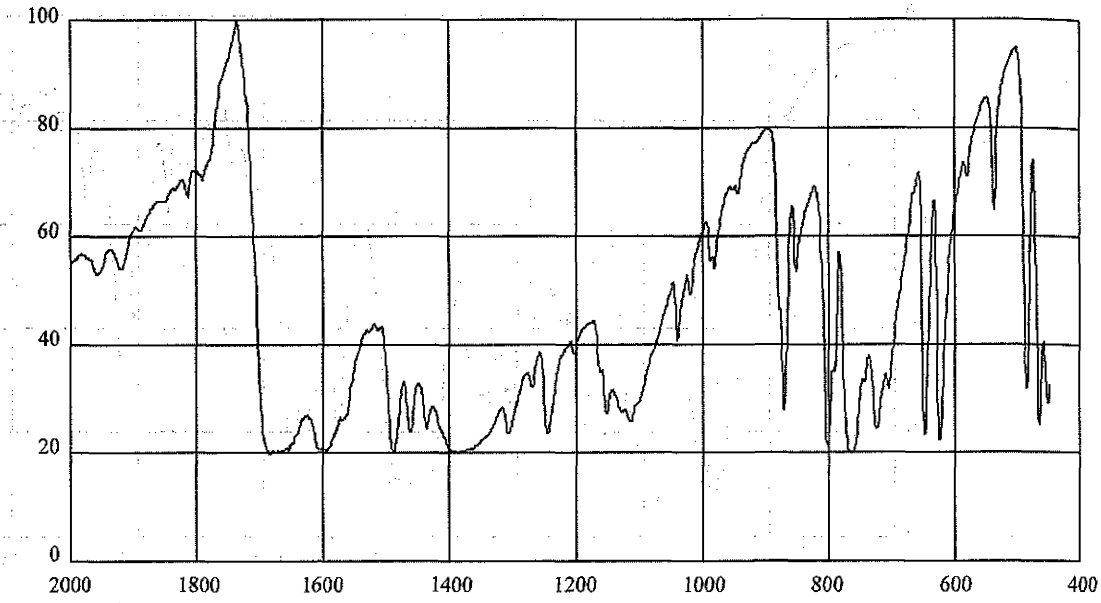
Captopril *FTIR* Phase: KBr disc



Carbamazepine

FTIR

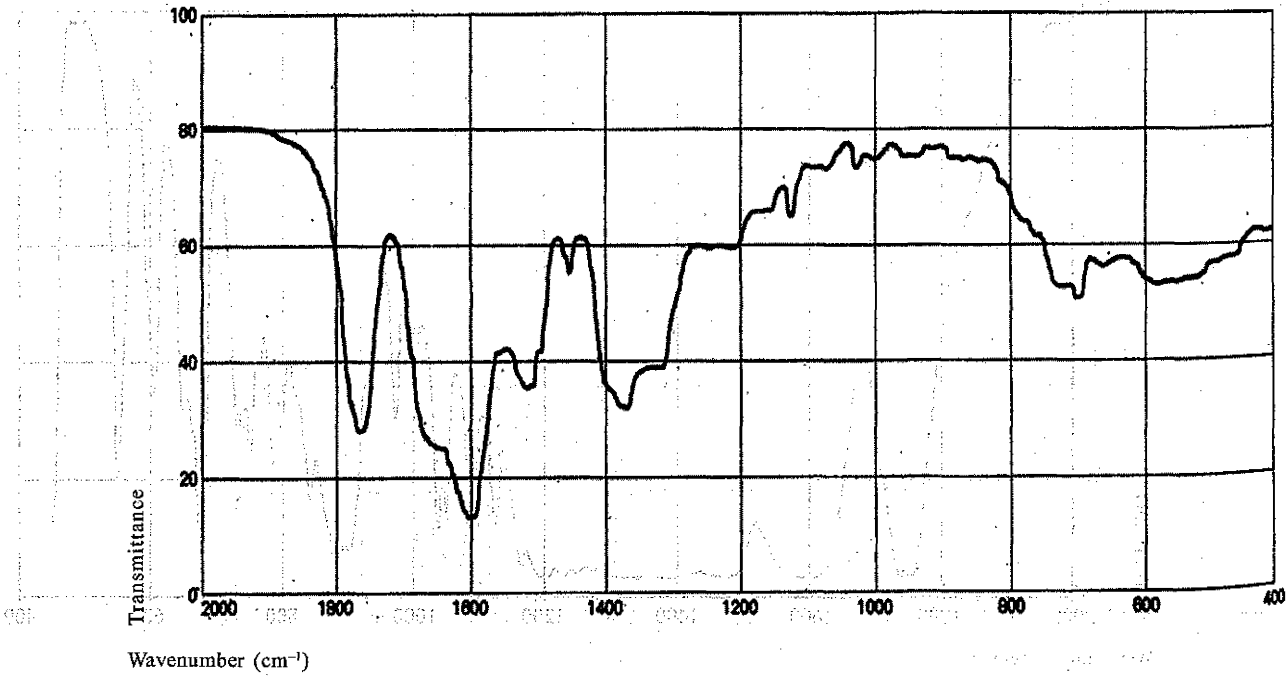
Phase: KBr disc



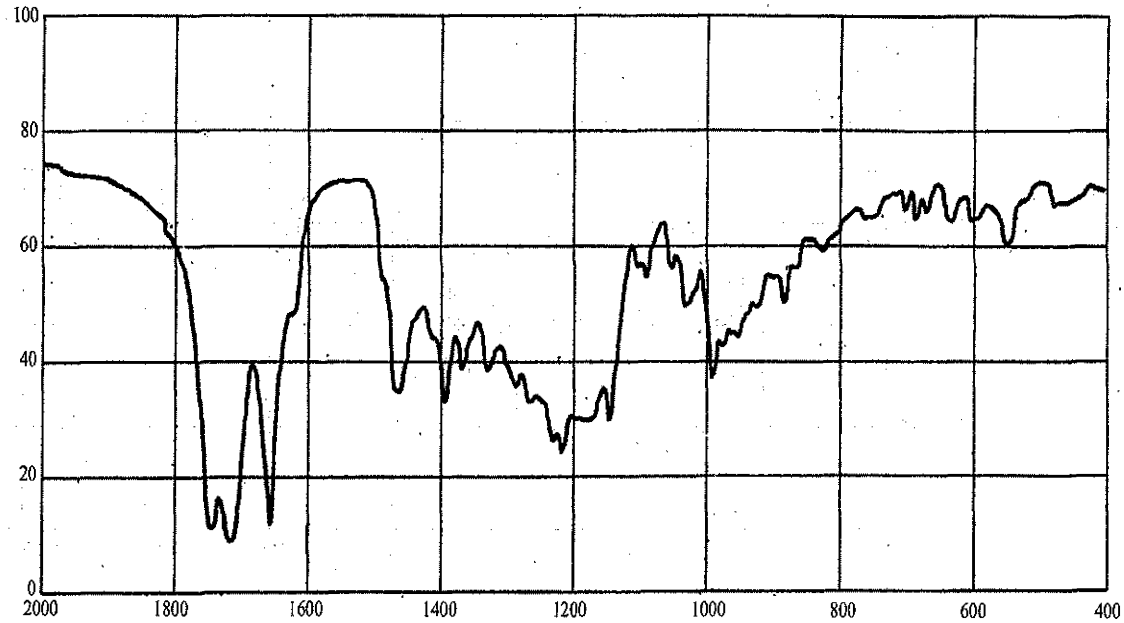
Carbenicillin Sodium

Dispersive IR

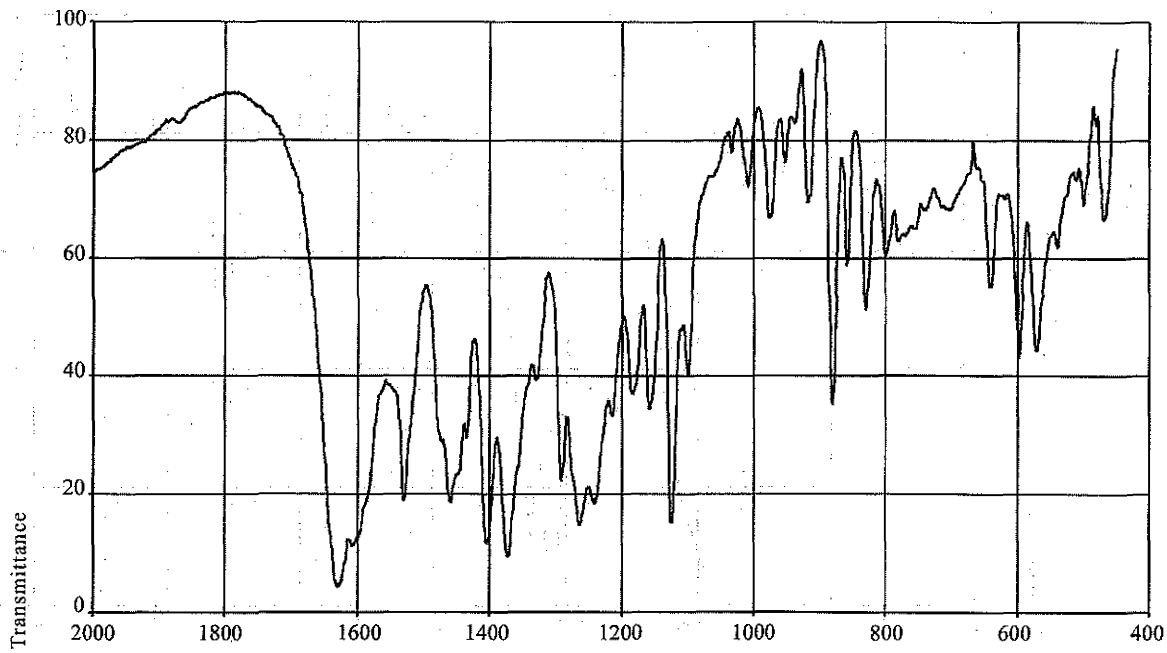
Phase: KBr disc



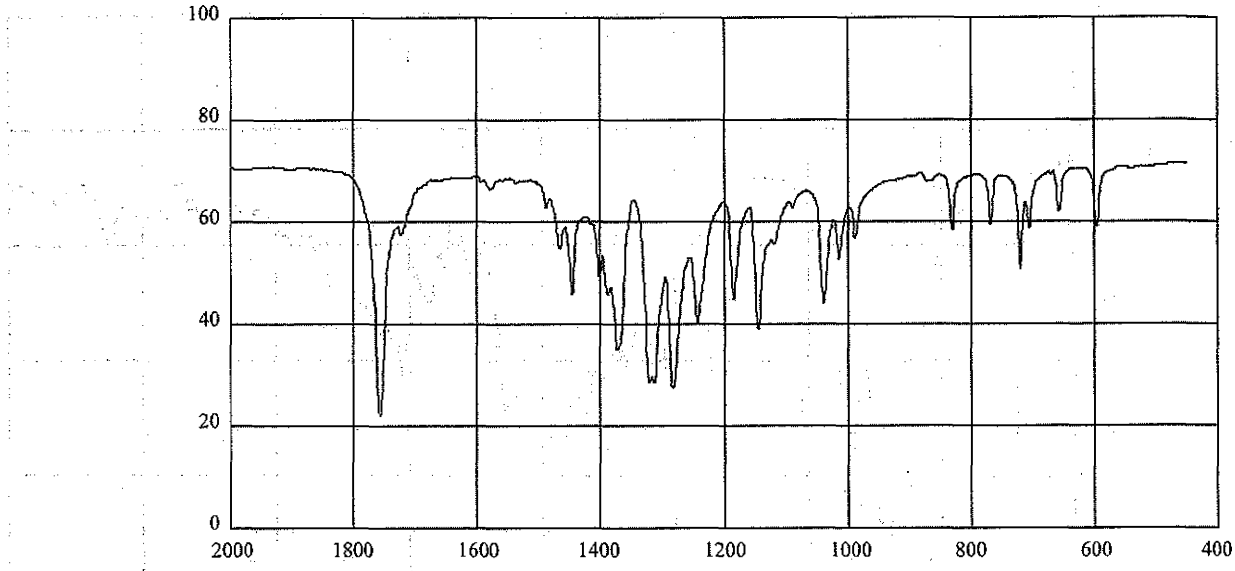
Carbenoxolone *Dispersive IR* Phase: KBr disc



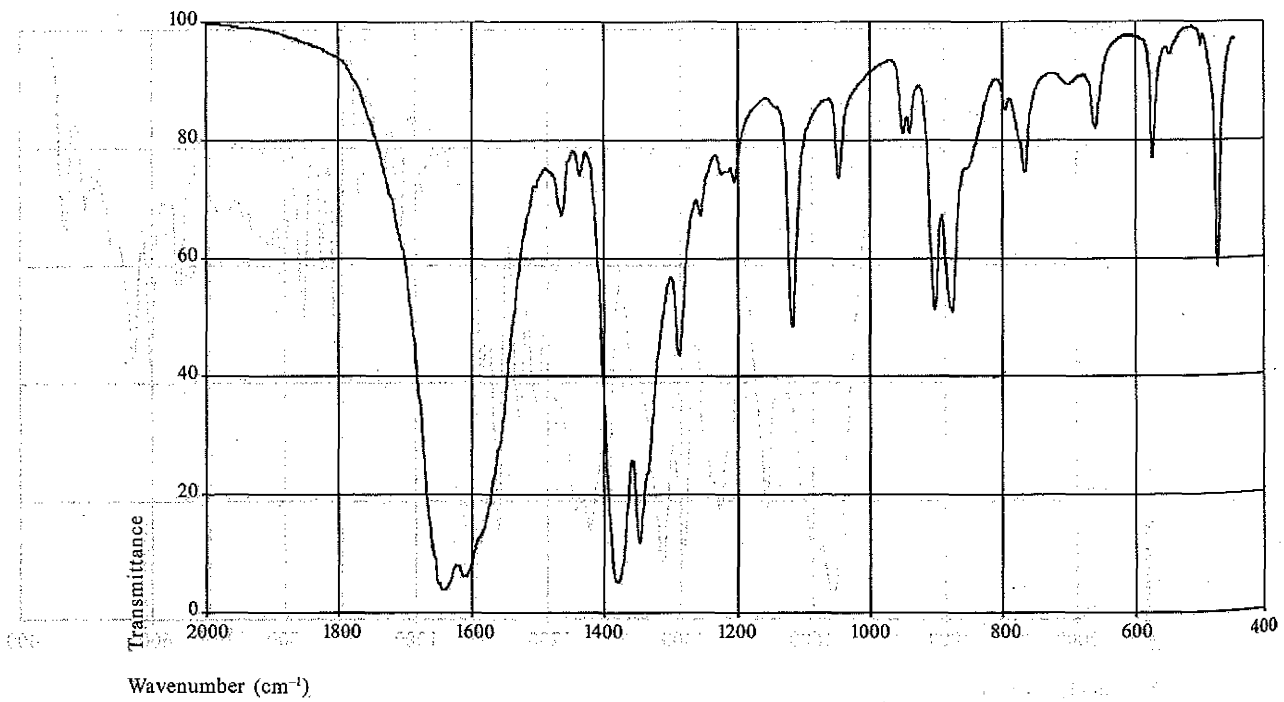
Carbidopa *FTIR* Phase: KBr disc



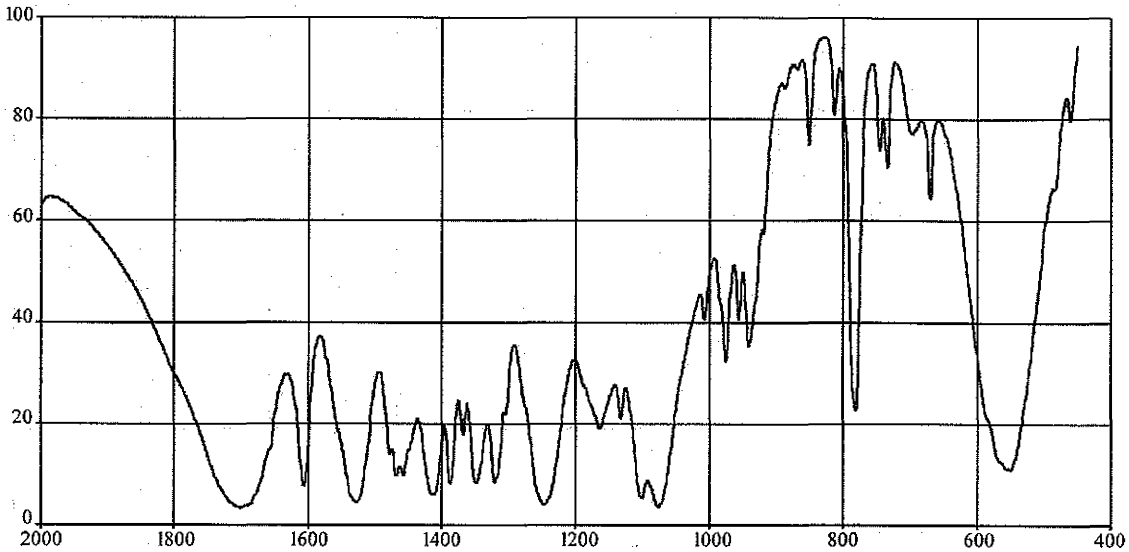
Carbimazole *FTIR* Phase: KBr disc



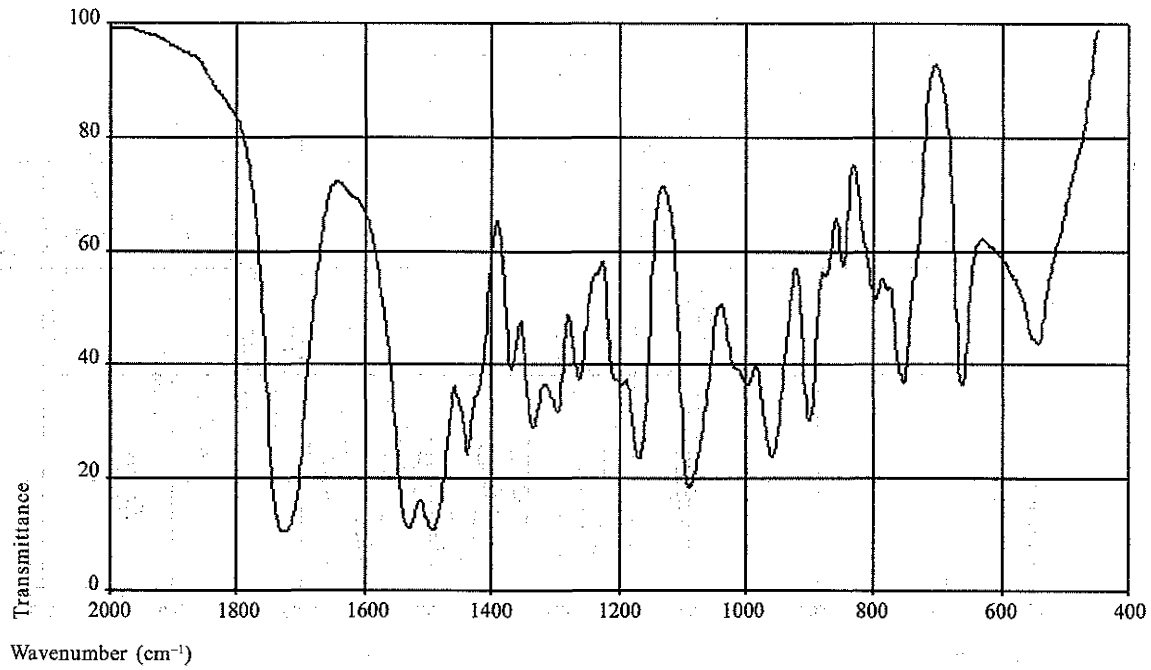
Carboplatin *FTIR* Phase: KBr disc



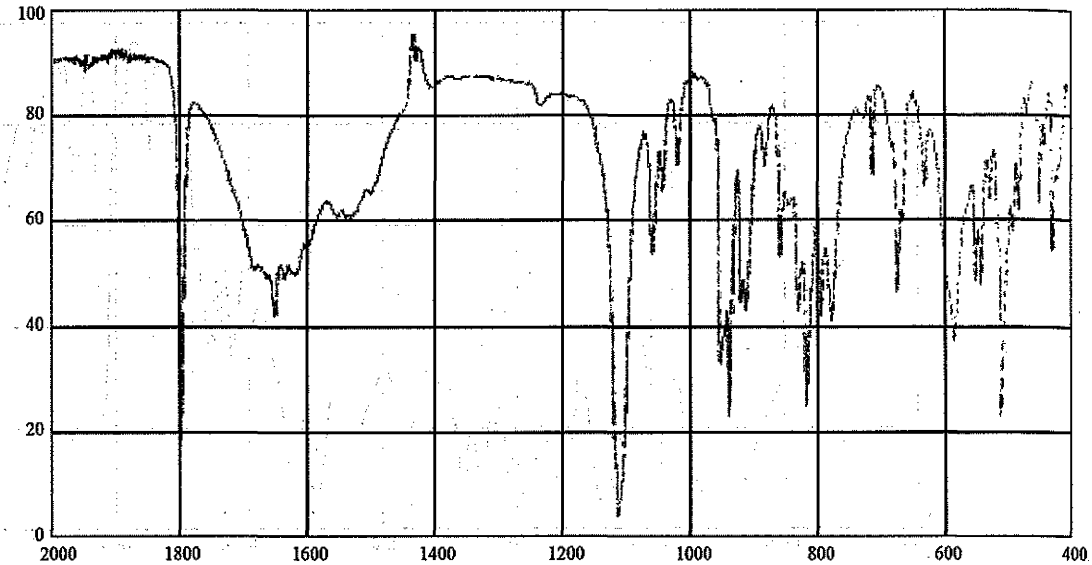
Carisoprodol *FTIR* Phase: KBr disc



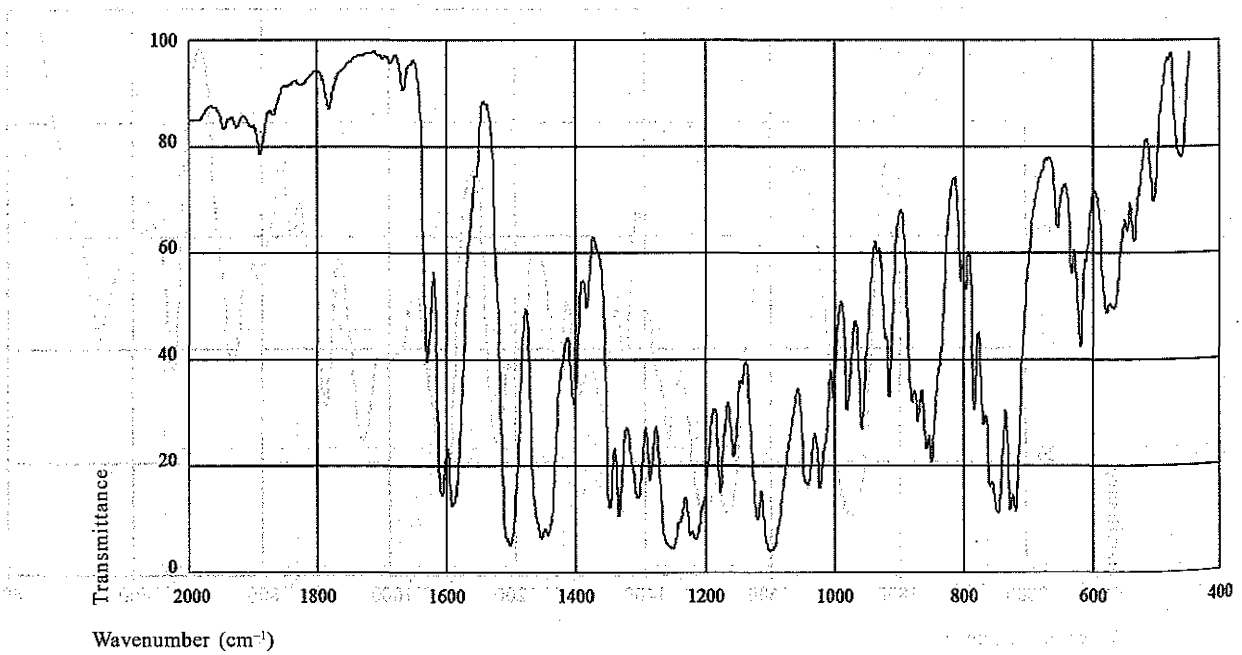
Carmustine *FTIR* Phase: KBr disc



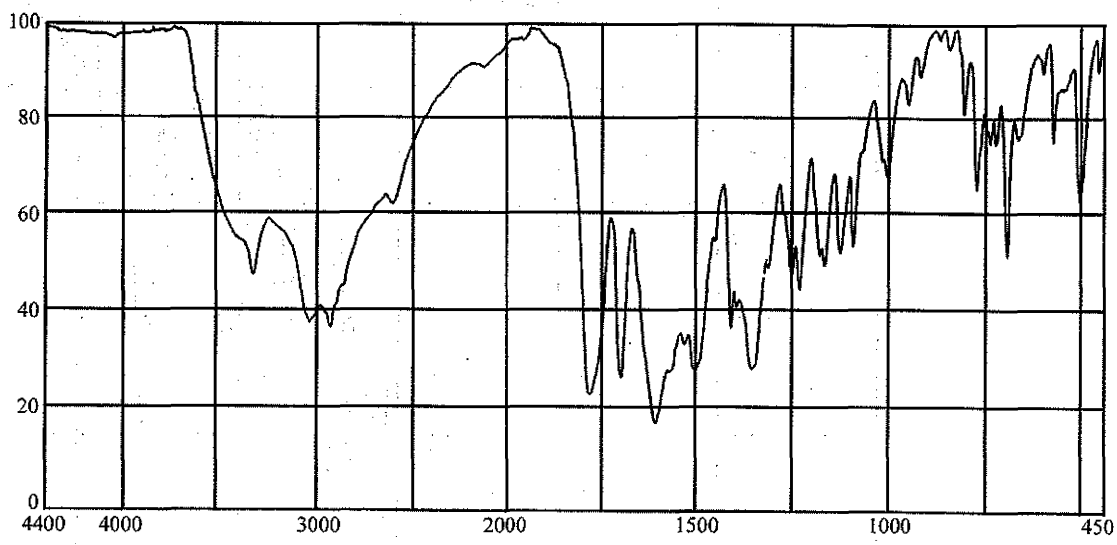
Carprofen *FTIR* Phase: KBr disc



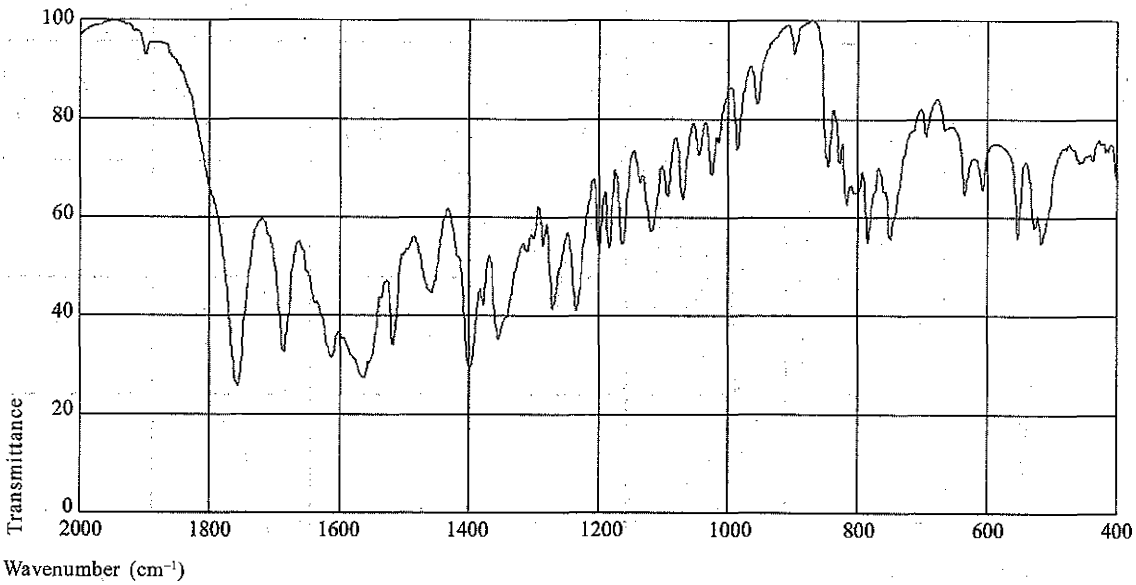
Carvedilol *FTIR* Phase: KBr disc



Cefaclor *FTIR* Phase: KBr disc



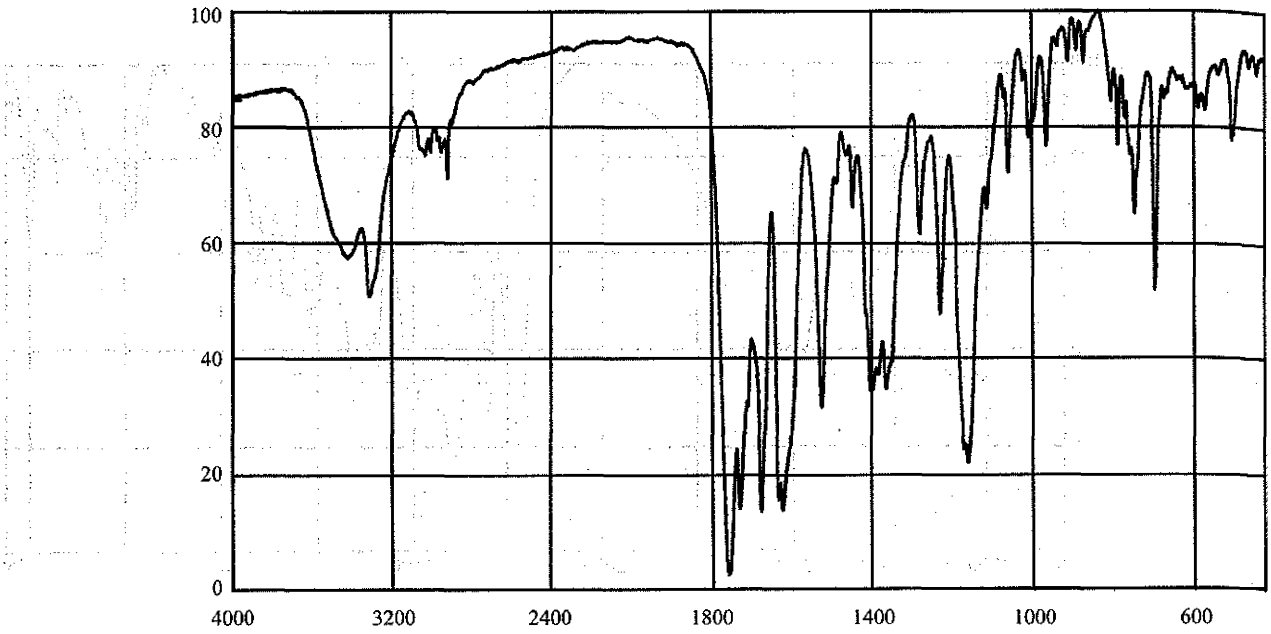
Cefadroxil Monohydrate *FTIR* Phase: KBr disc



Cefamandole Nafate

FTIR

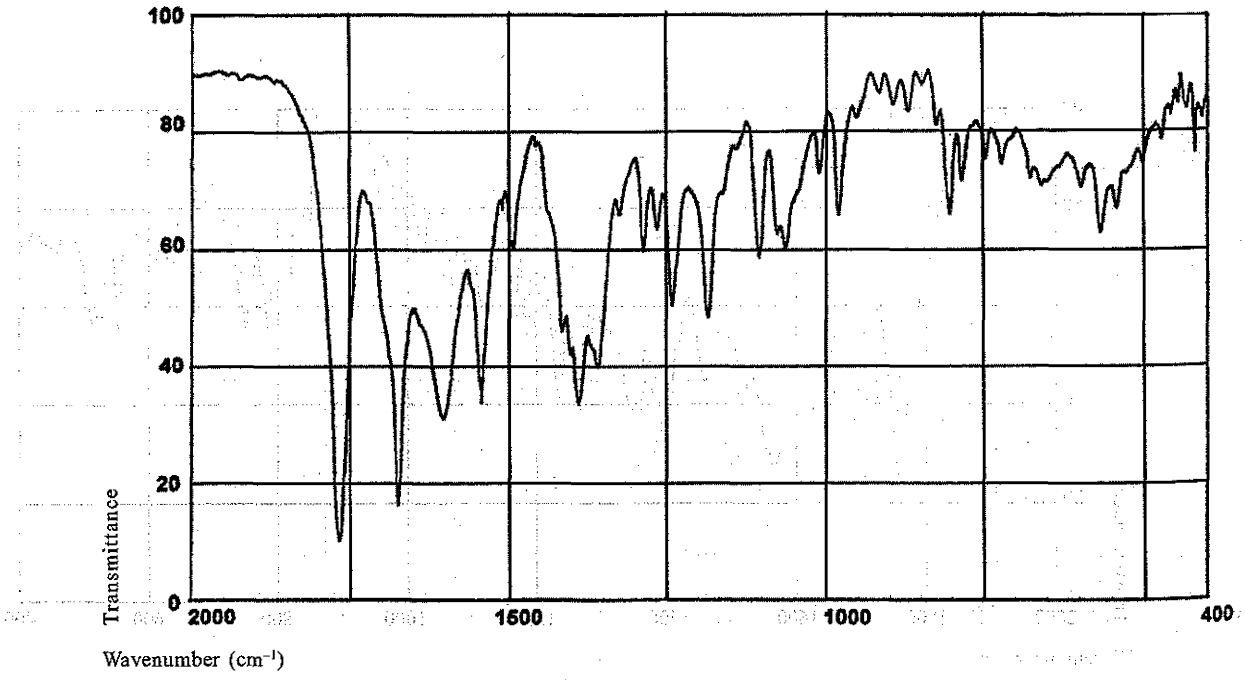
Phase: KBr disc



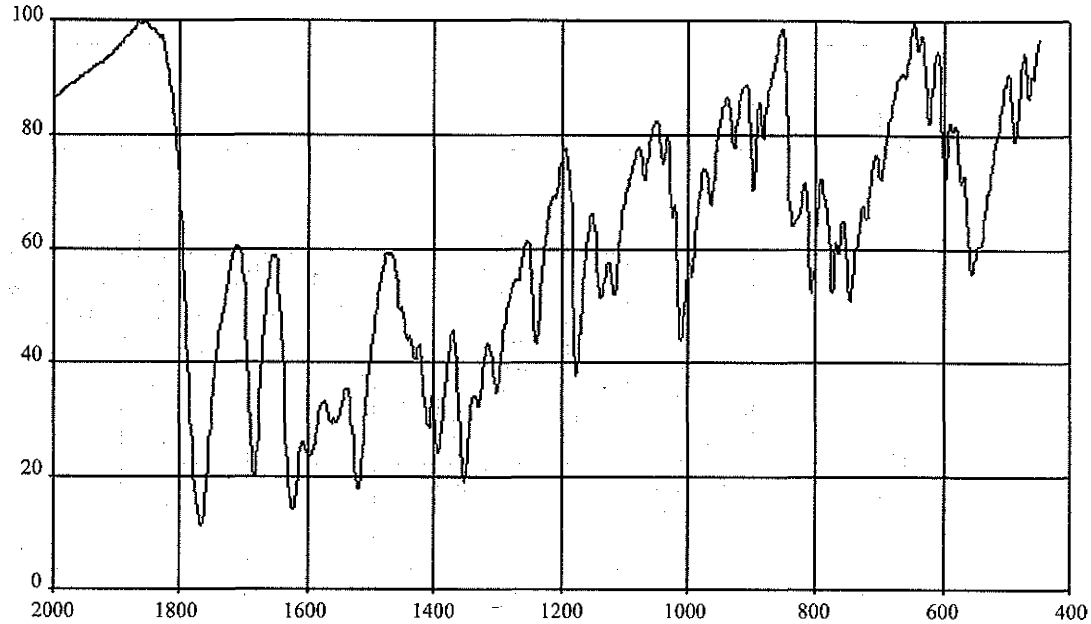
Cefazolin Sodium

FTIR

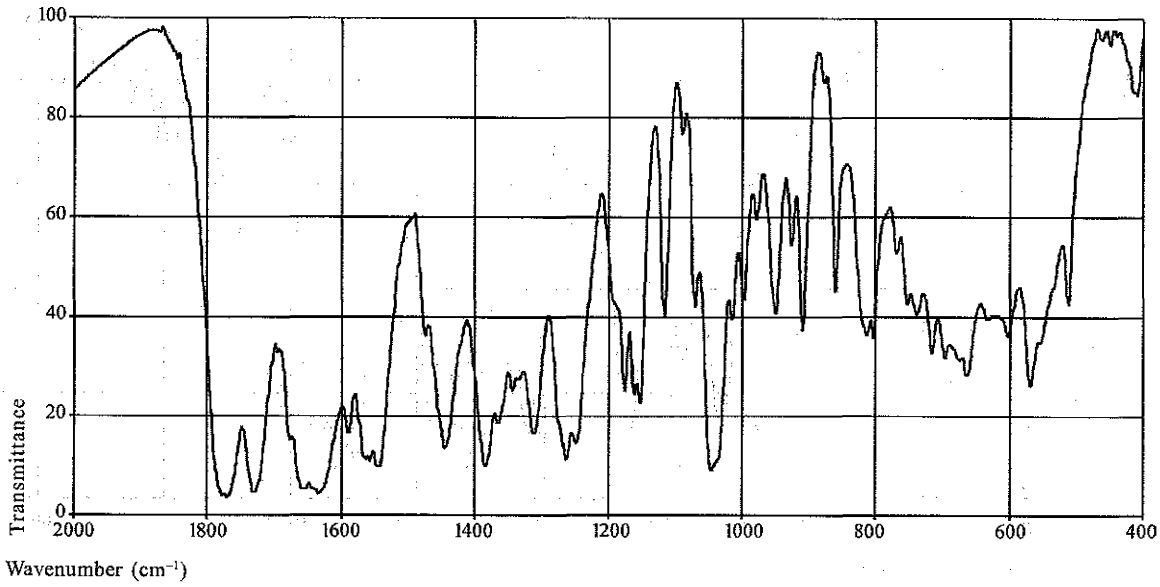
Phase: KBr disc



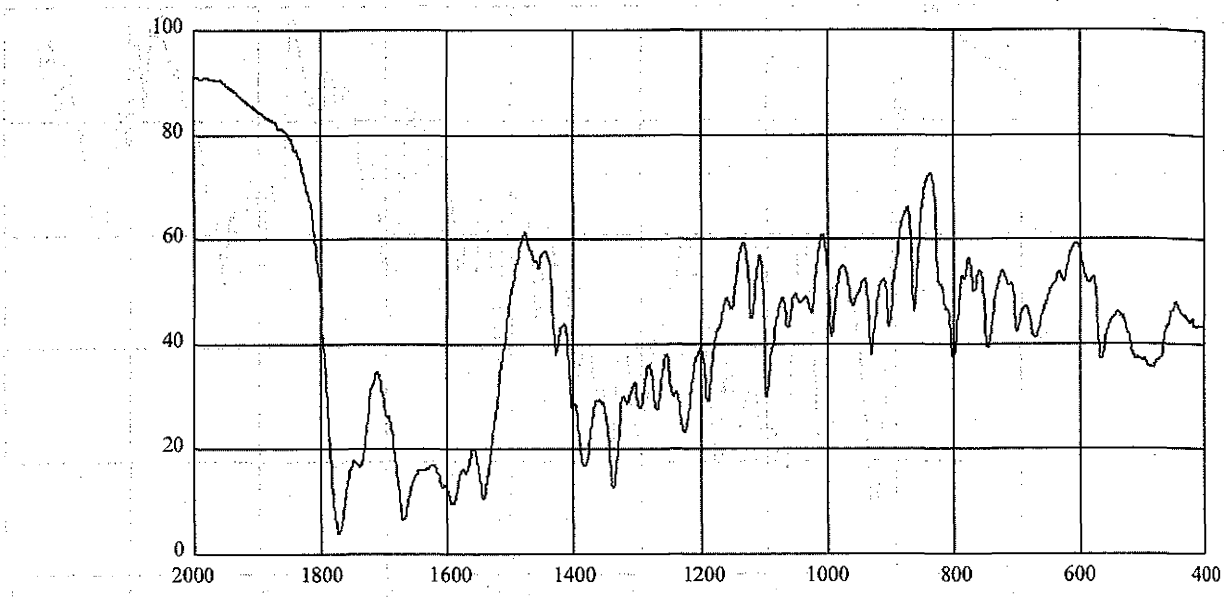
Cefdinir *FTIR* Phase: KBr disc



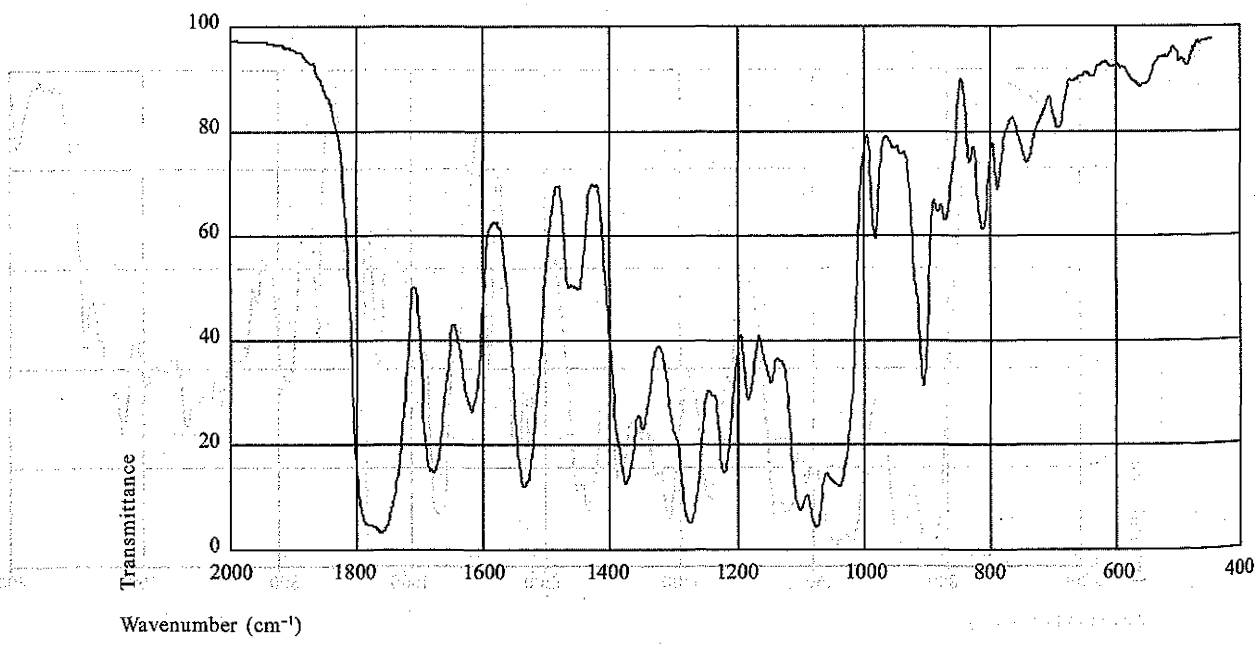
Cefepime Hydrochloride *FTIR* Phase: KBr disc



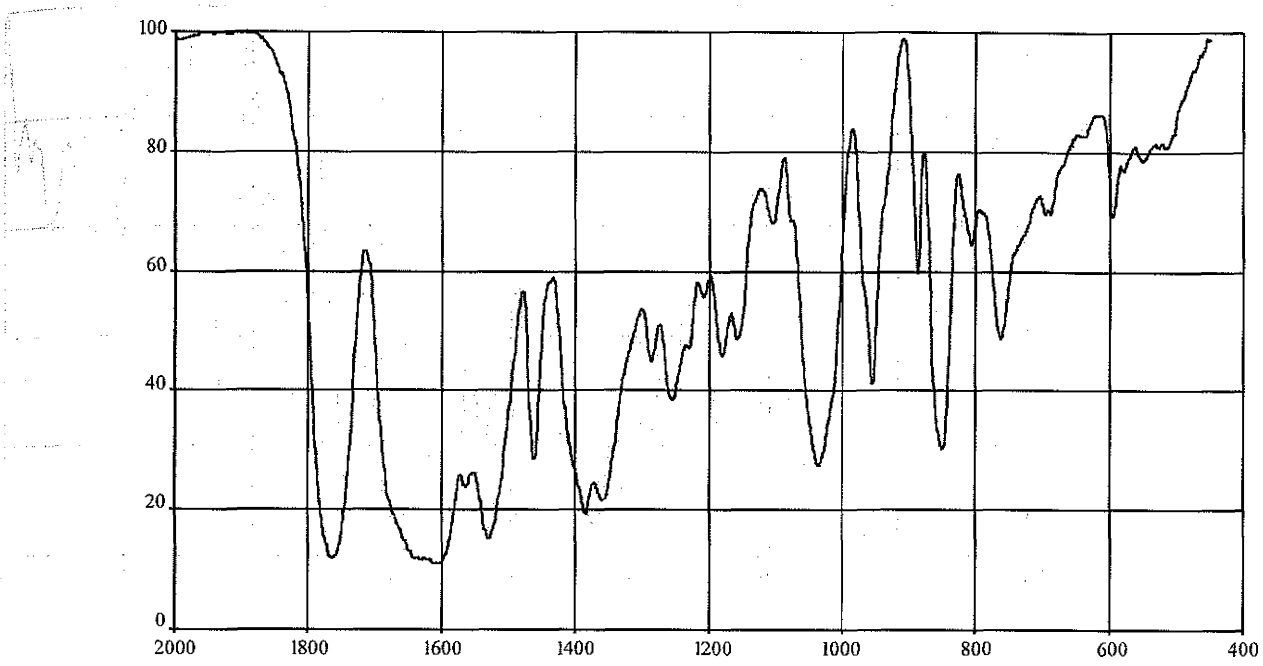
Cefixime *FTIR* Phase: KBr disc



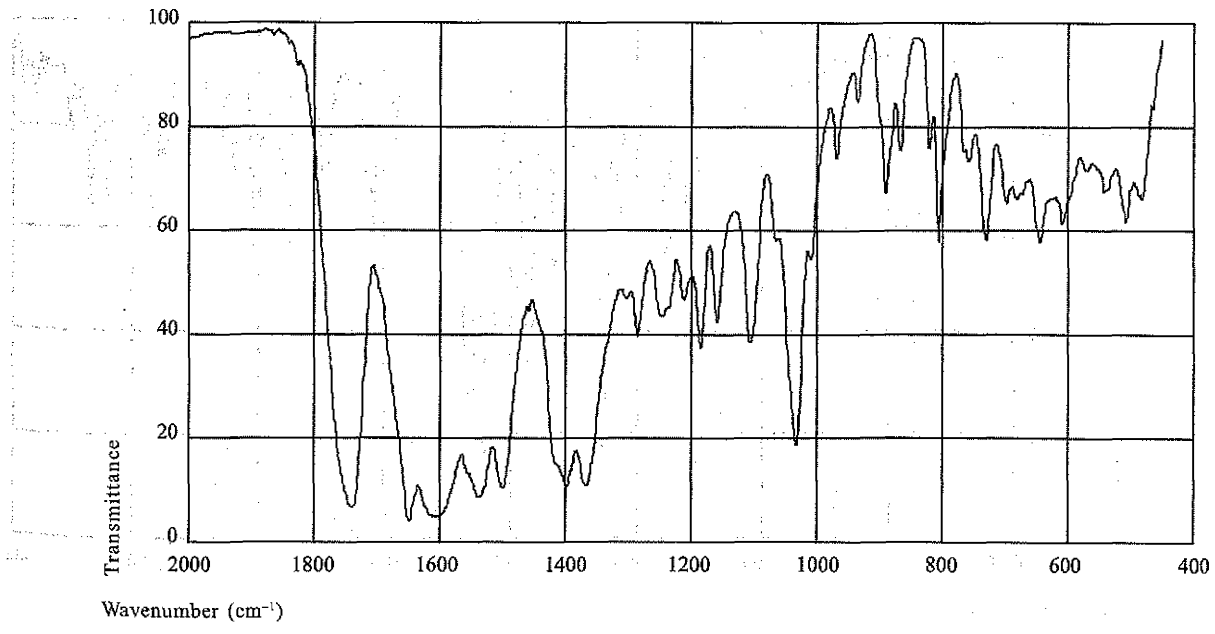
Cefpodoxime Proxetil *FTIR* Phase: KBr disc



Ceftiofur Sodium *FTIR* Phase: KBr disc



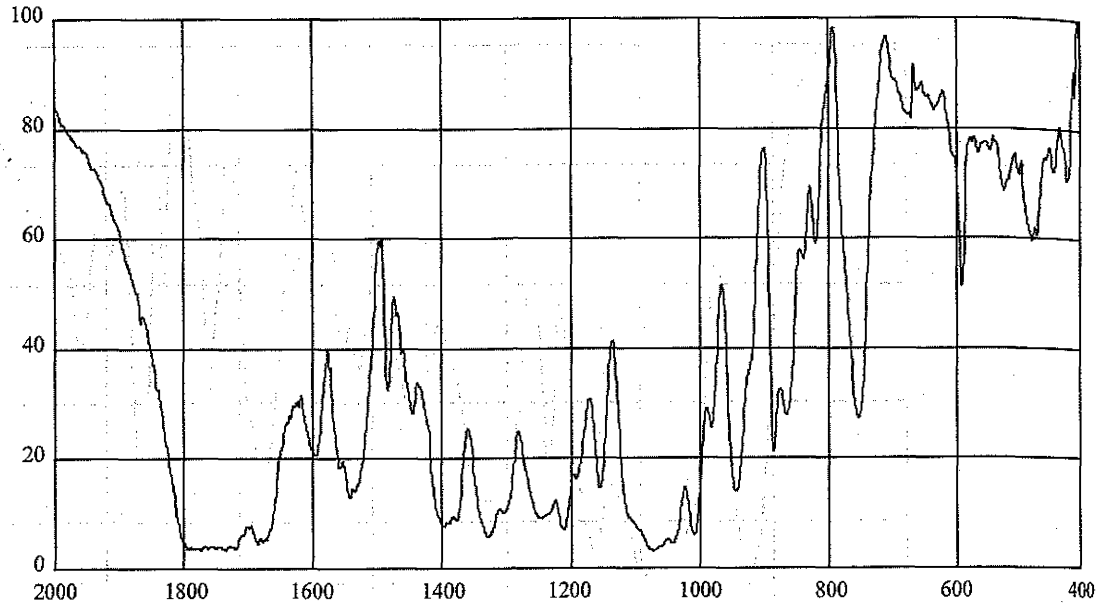
Ceftriaxone Sodium *FTIR* Phase: KBr disc



Cefuroxime Axetil

FTIR

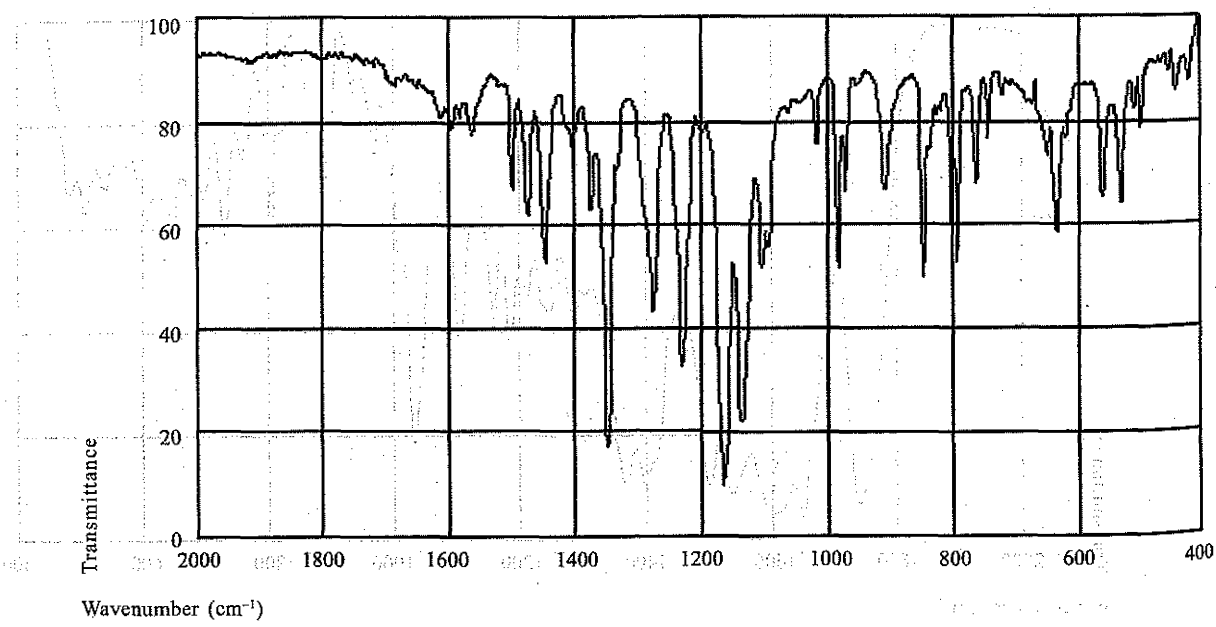
Phase: KBr disc



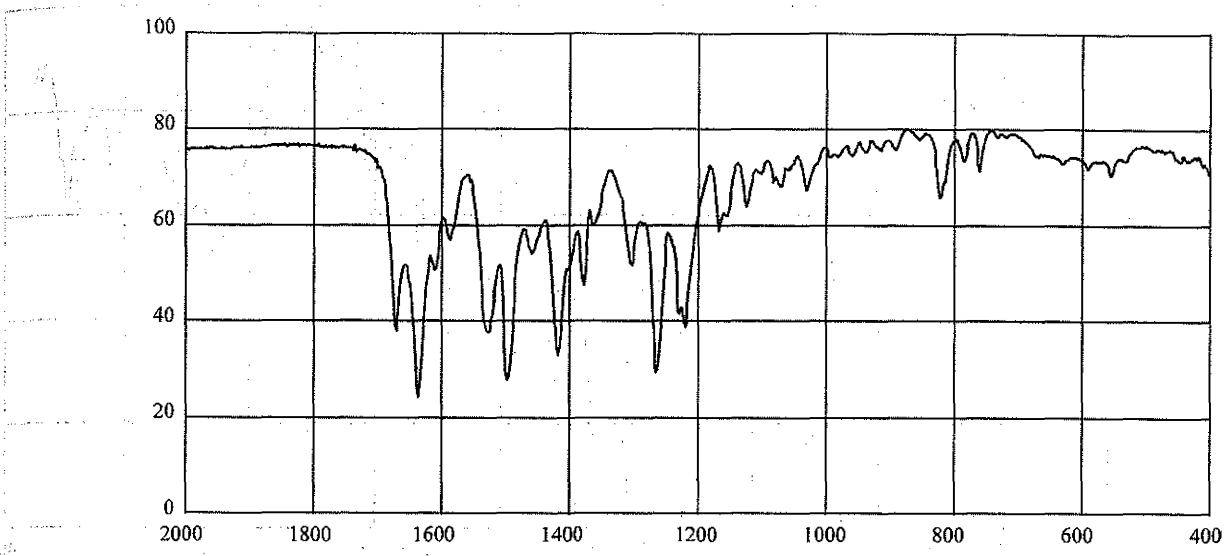
Celecoxib

FTIR

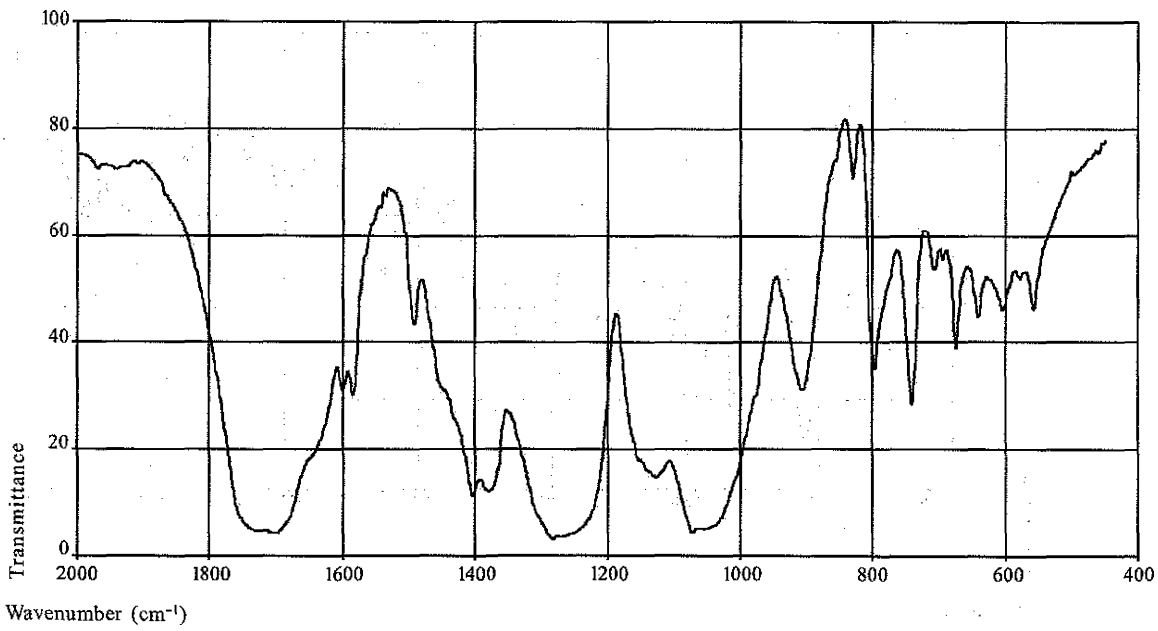
Phase: KBr disc



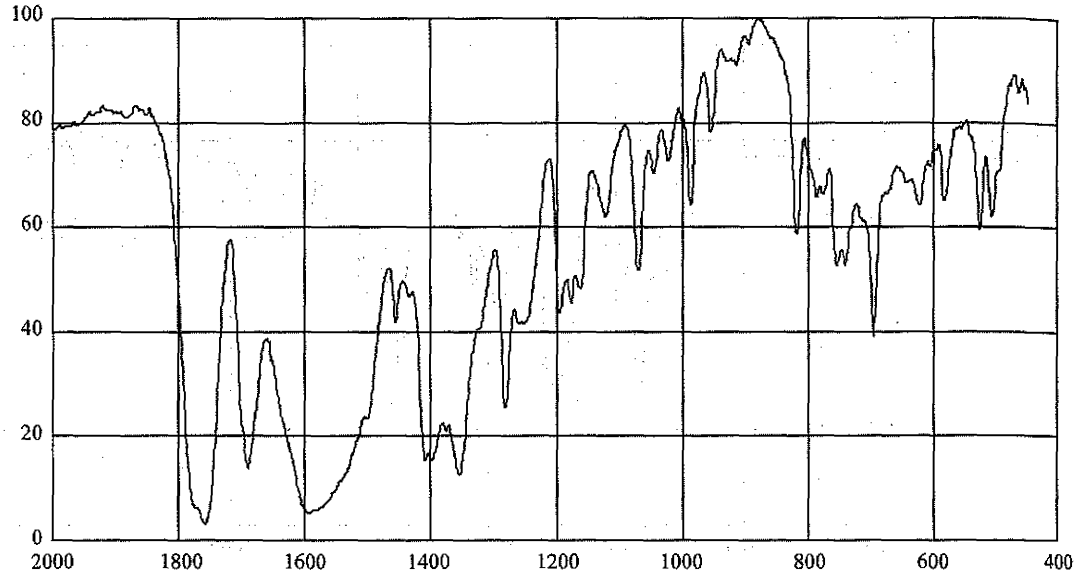
Celiprolol Hydrochloride *FTIR* Phase: KBr disc



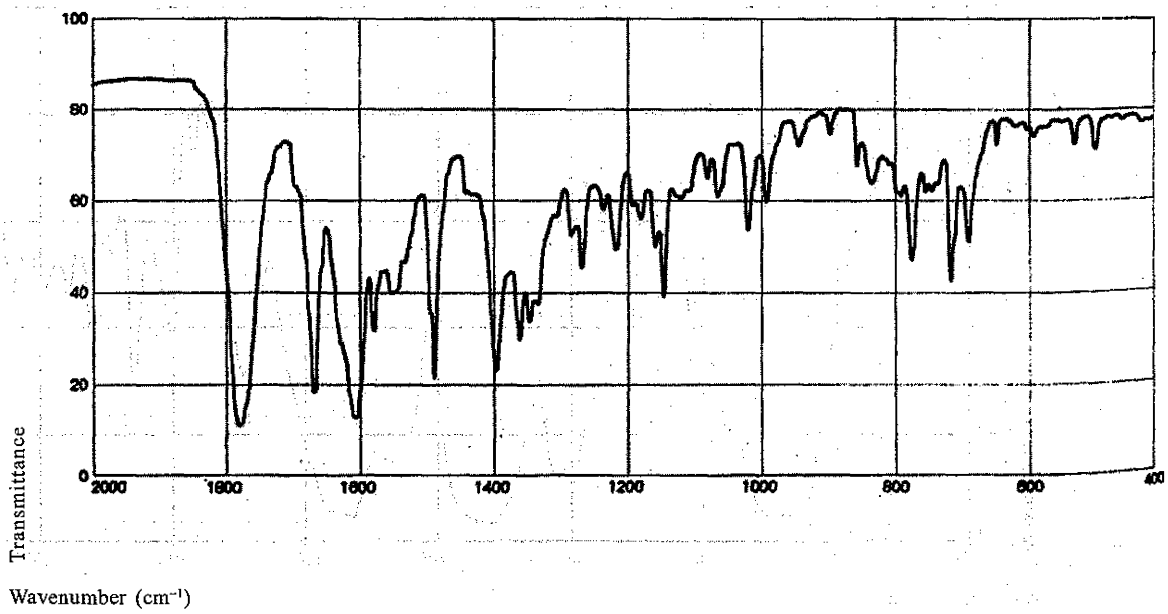
Cellulose Acetate Phthalate *FTIR* Phase: KBr disc



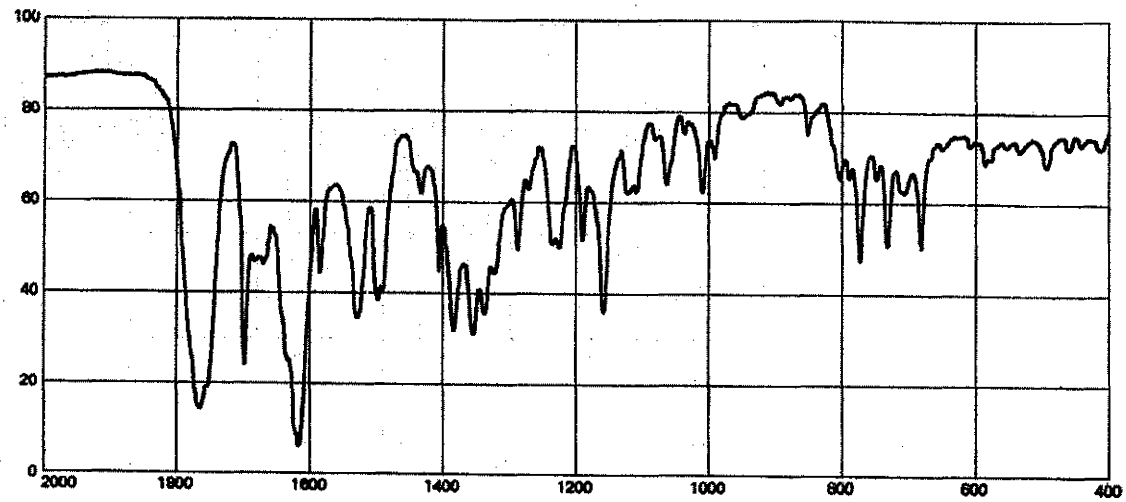
Cephalexin *FTIR* Phase: KBr disc



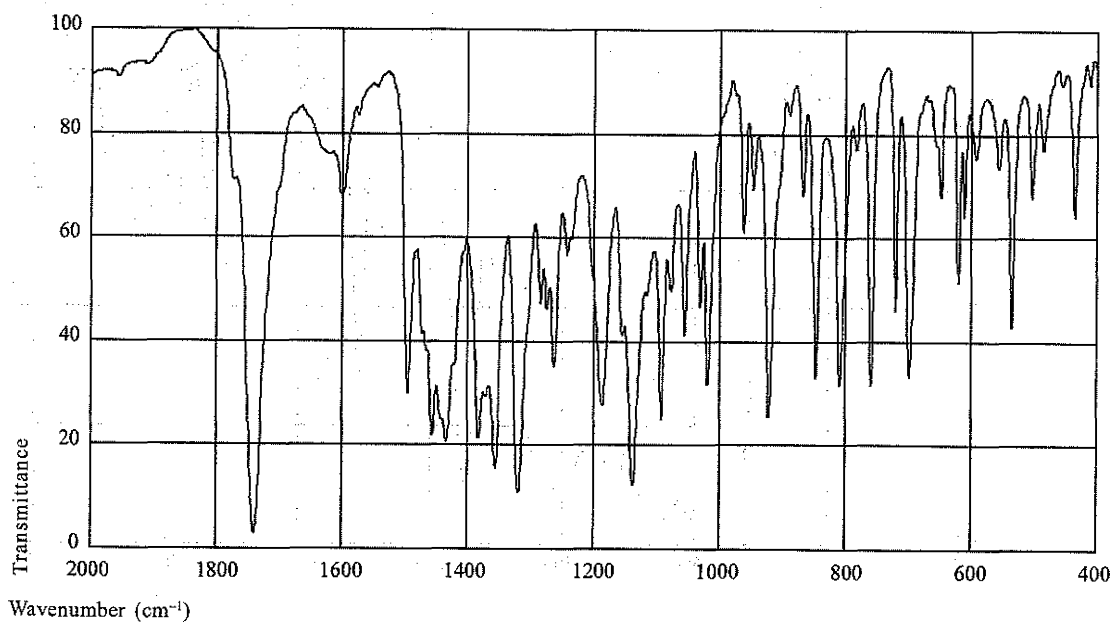
Cephaloridine (α form) *Dispersive IR* Phase: KBr disc



Cephaloridine (δ form) *Dispersive IR* Phase: KBr disc

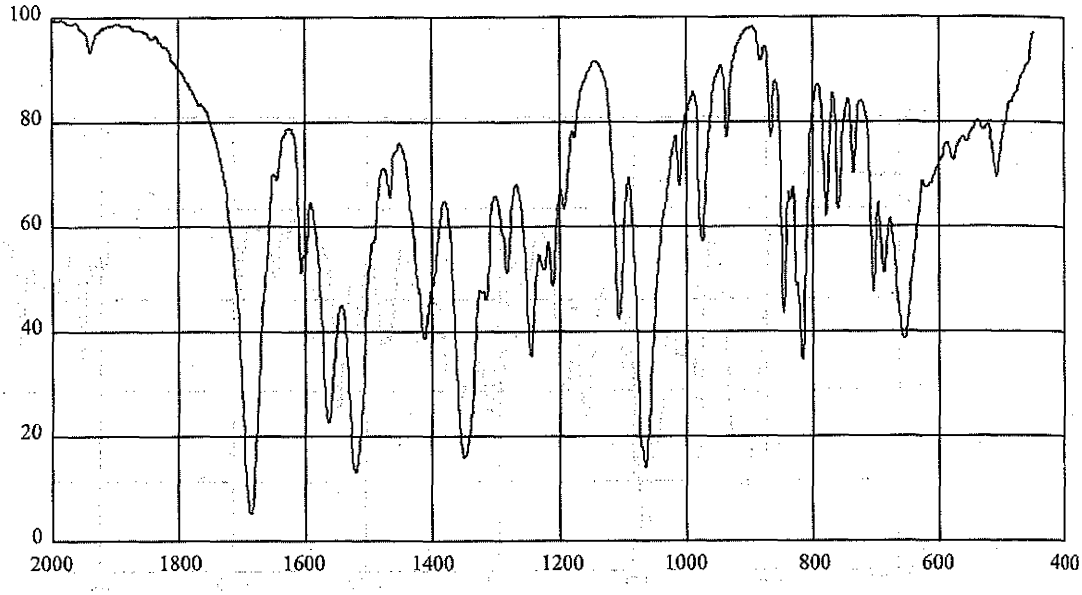


Cetirizine Hydrochloride *FTIR* Phase: KBr disc



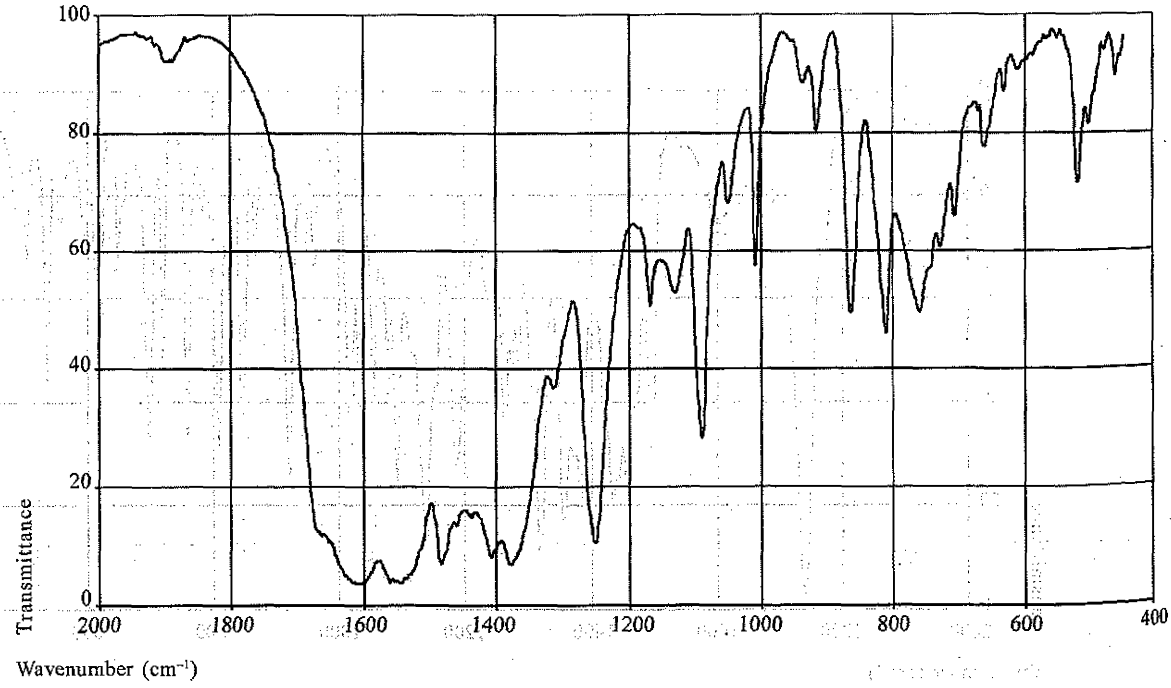
Chloramphenicol

FTIR Phase: KBr disc

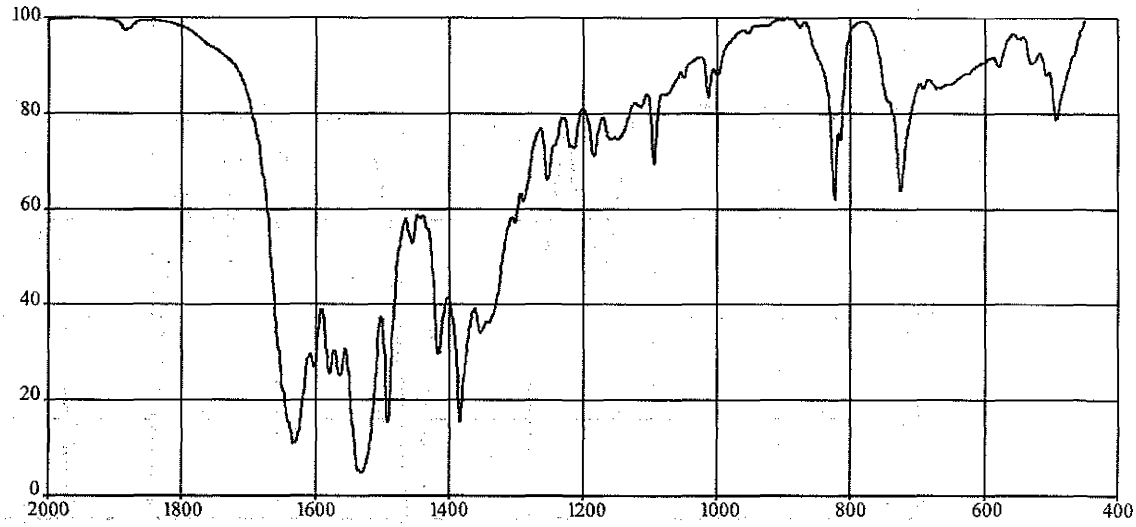


Chlorhexidine

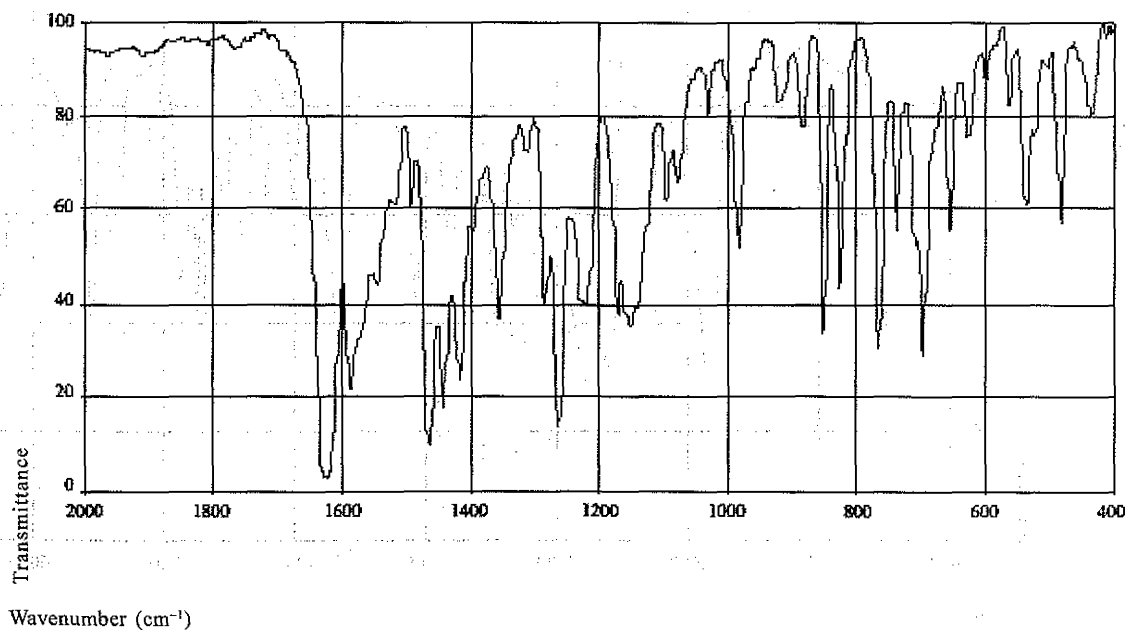
FTIR Phase: KBr disc



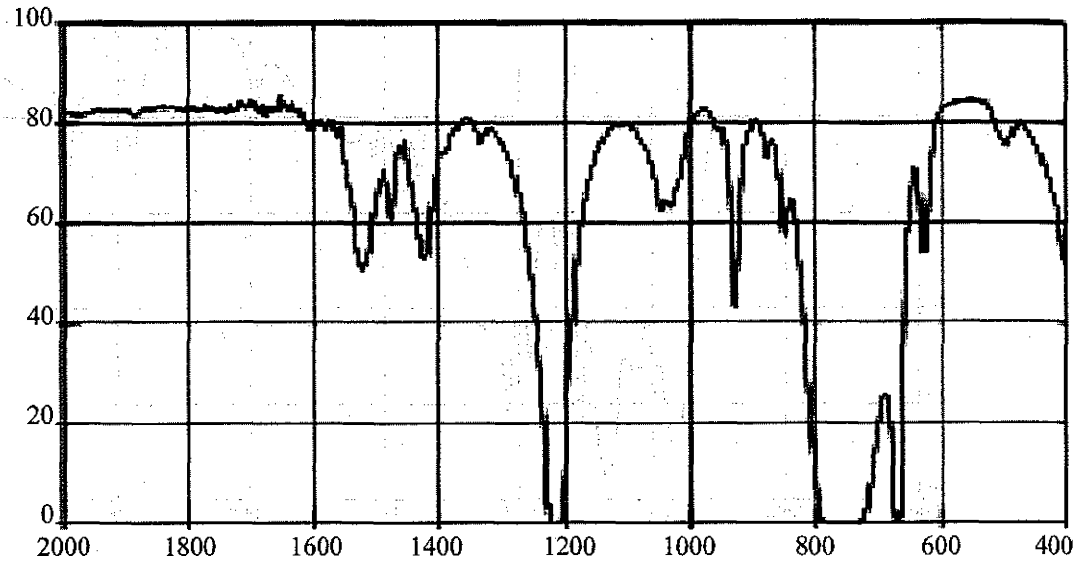
Chlorhexidine Hydrochloride *Dispersive IR* Phase: KBr disc



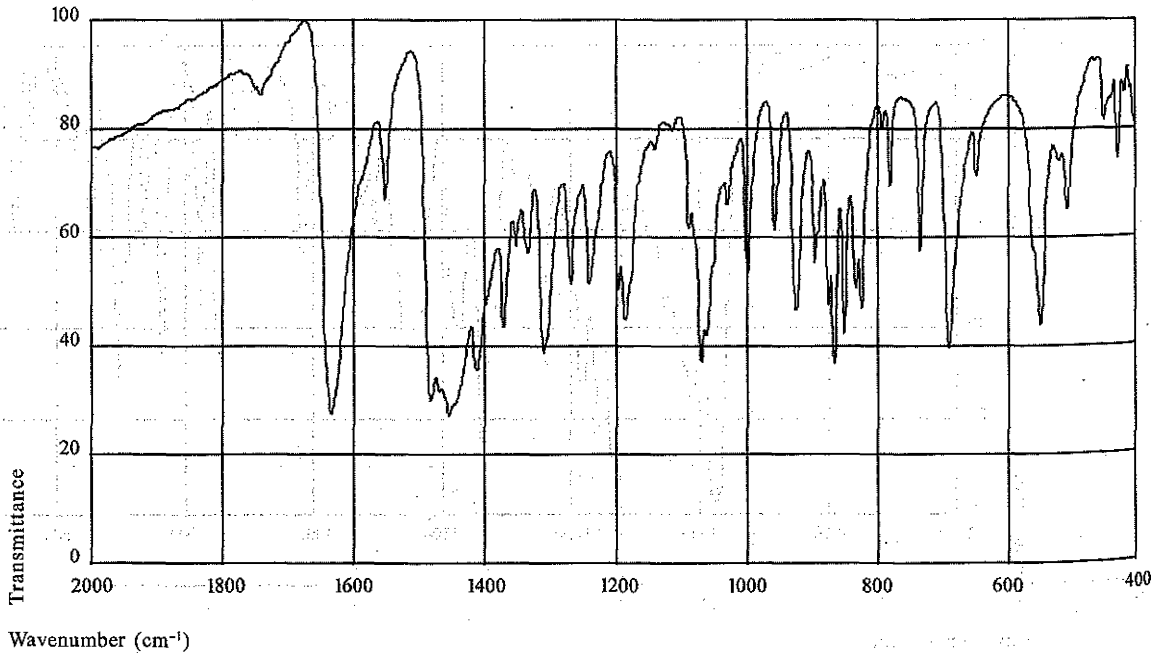
Chlordiazepoxide *FTIR* Phase: KBr disc



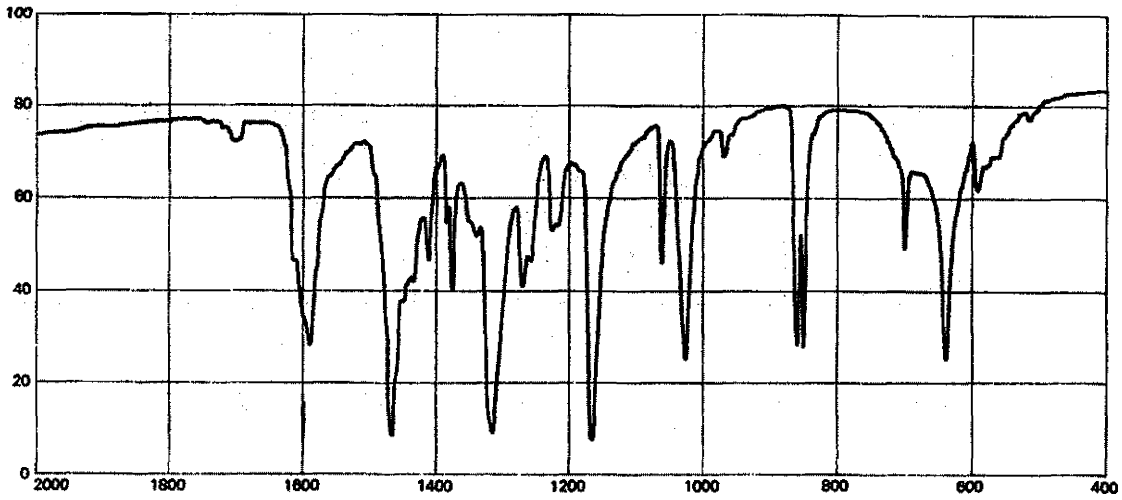
Chloroform *Dispersive IR* Phase: KBr disc



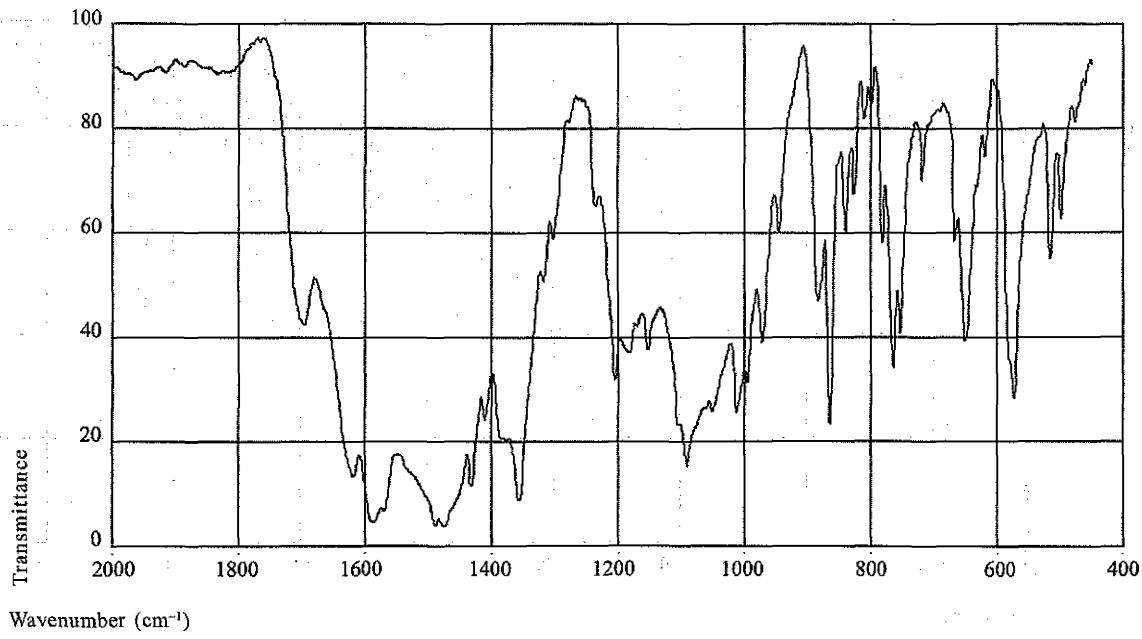
Chloroquine *FTIR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



Chloroxylenol *Dispersive IR* Phase: KBr disc

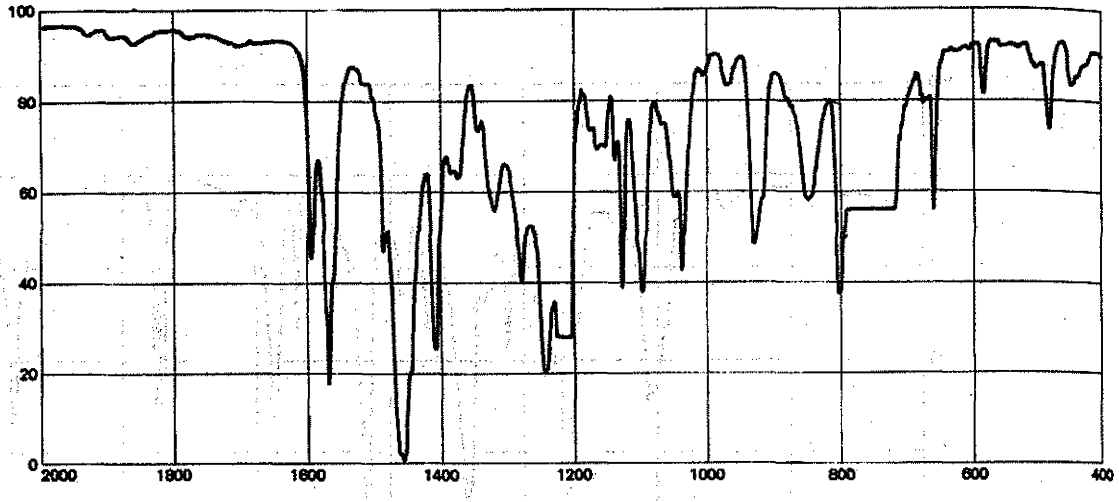


Chlorpheniramine Maleate *FTIR* Phase: KBr disc



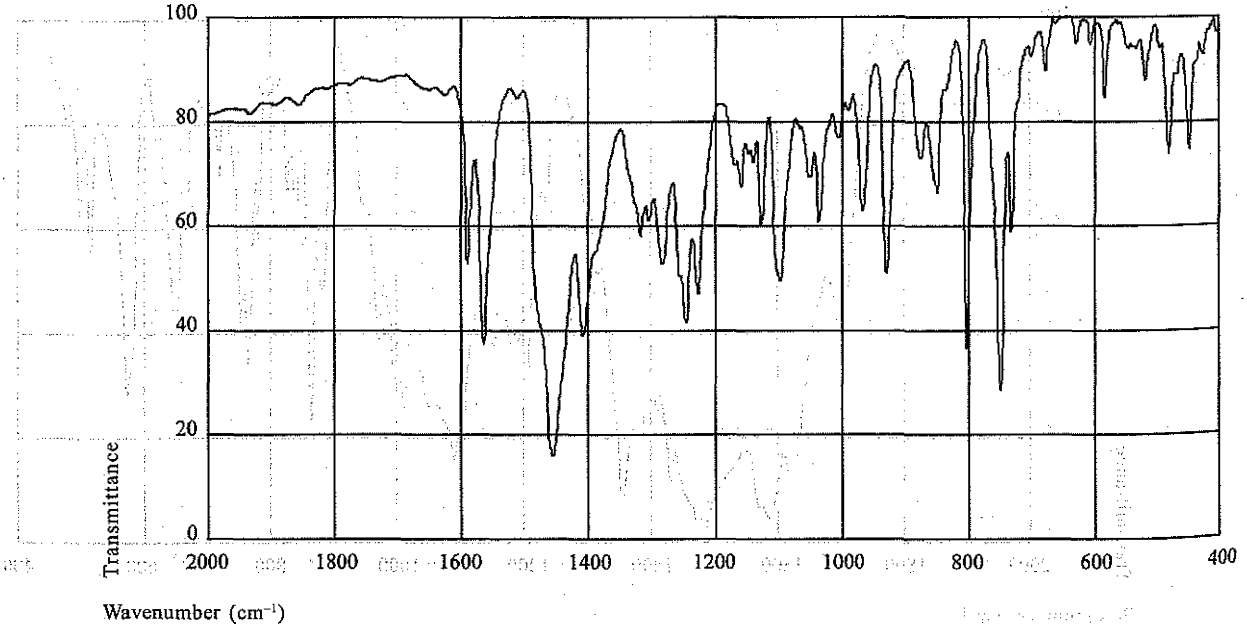
Chlorpromazine

Dispersive IR Phase: 5 per cent solution in $CHCl_3$ Thickness 0.1 mm

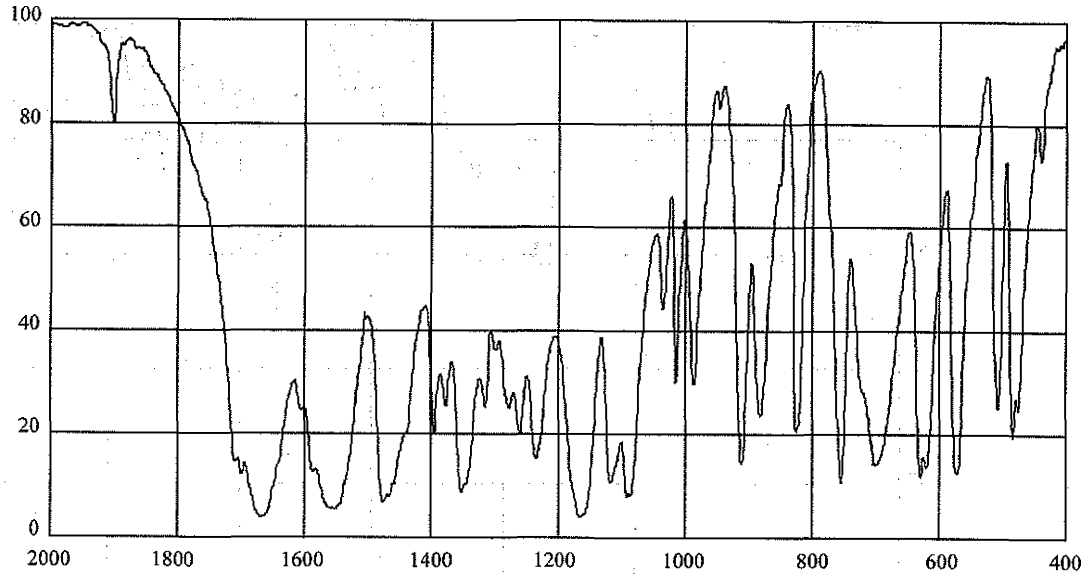


Chlorpromazine Hydrochloride

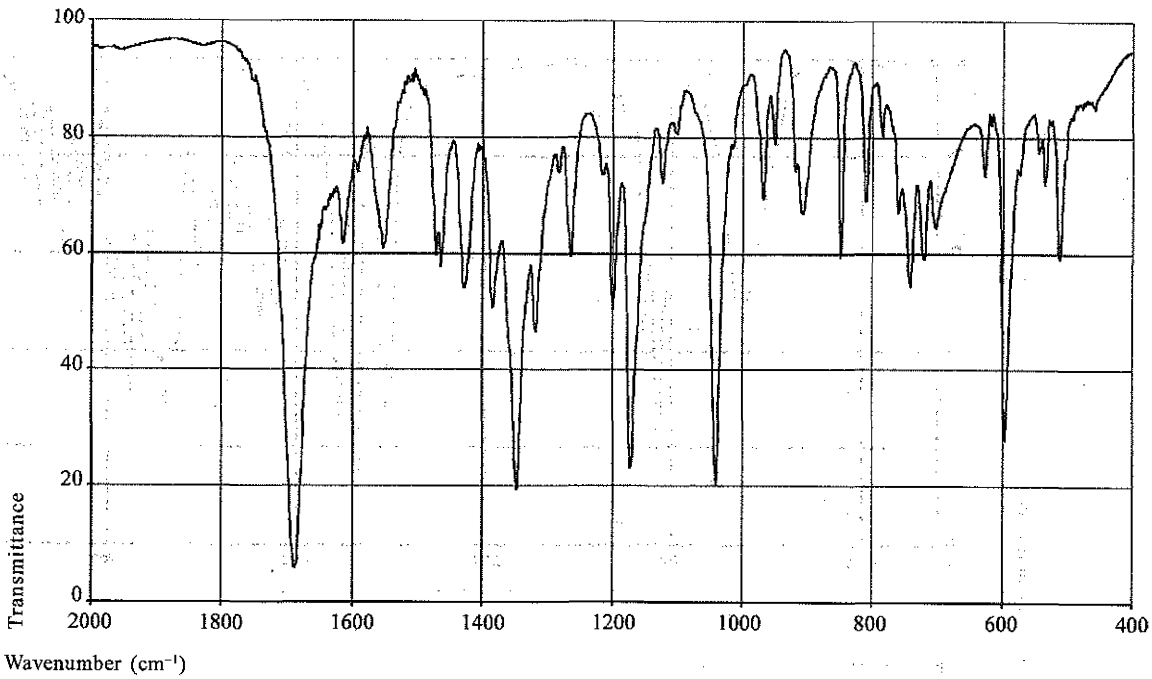
FTIR Phase: KBr disc



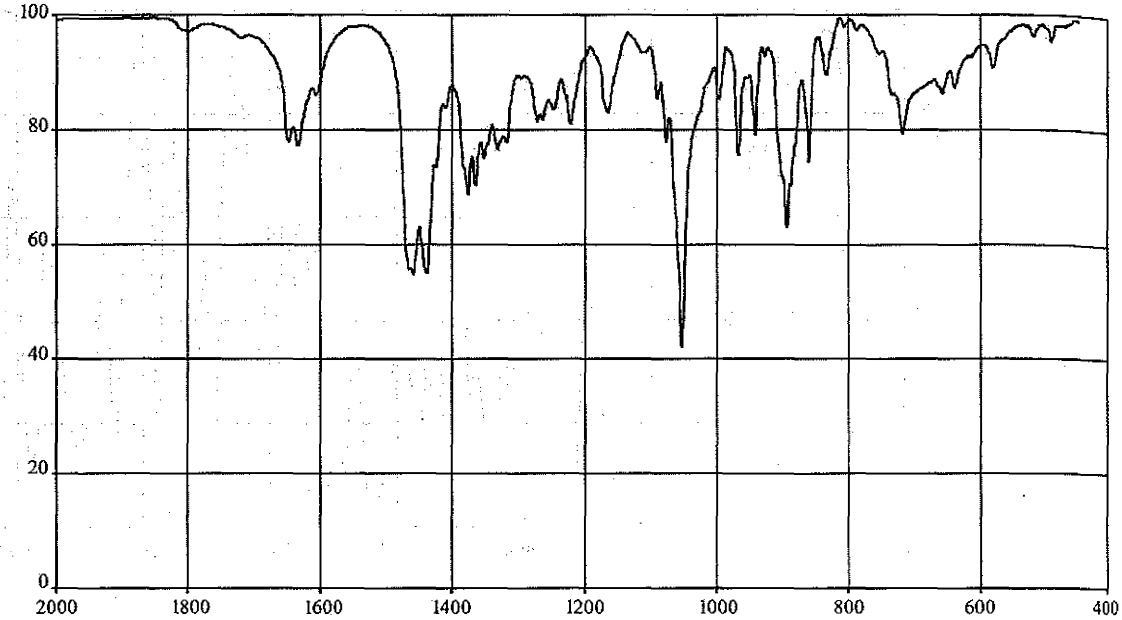
Chlorpropamide *FTIR* Phase: KBr disc



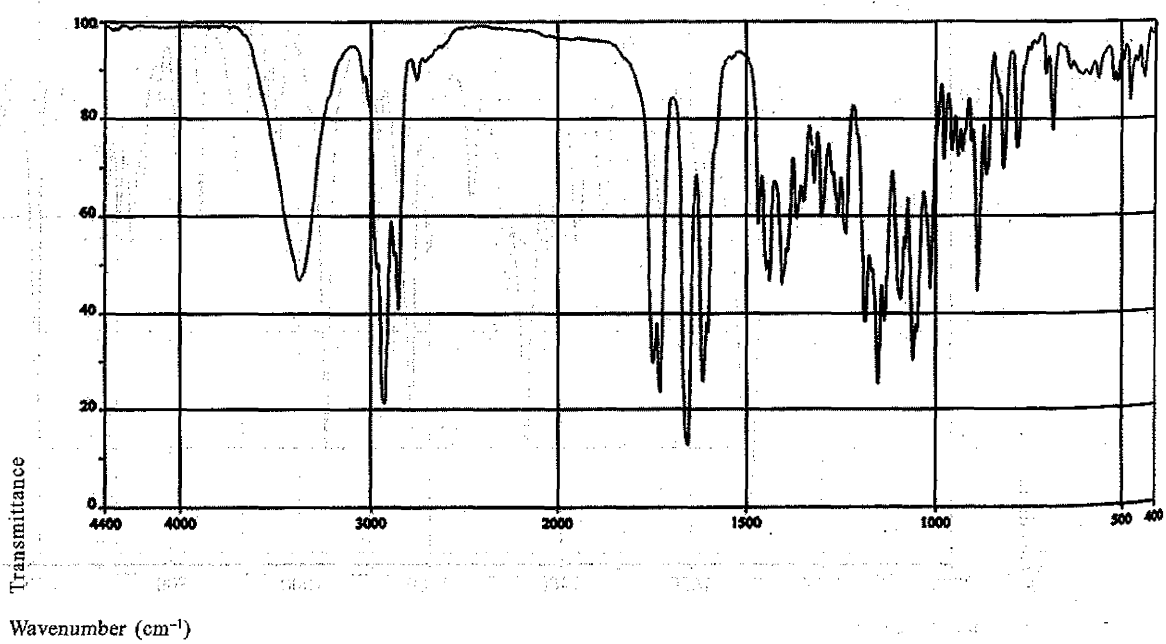
Chlorthalidone *FTIR* Phase: KBr disc



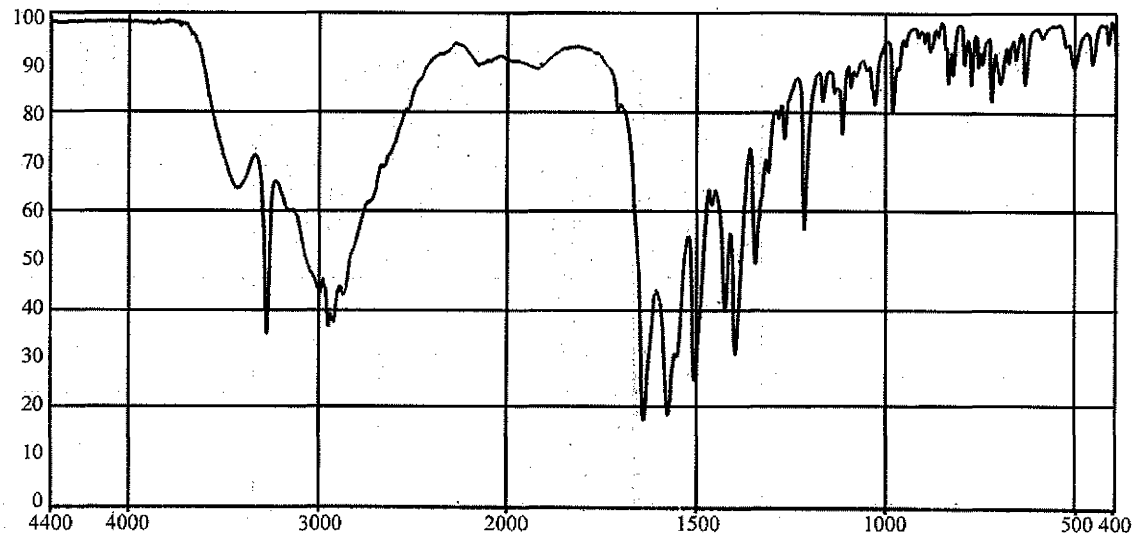
Cholecalciferol *Dispersive IR* Phase: KBr disc



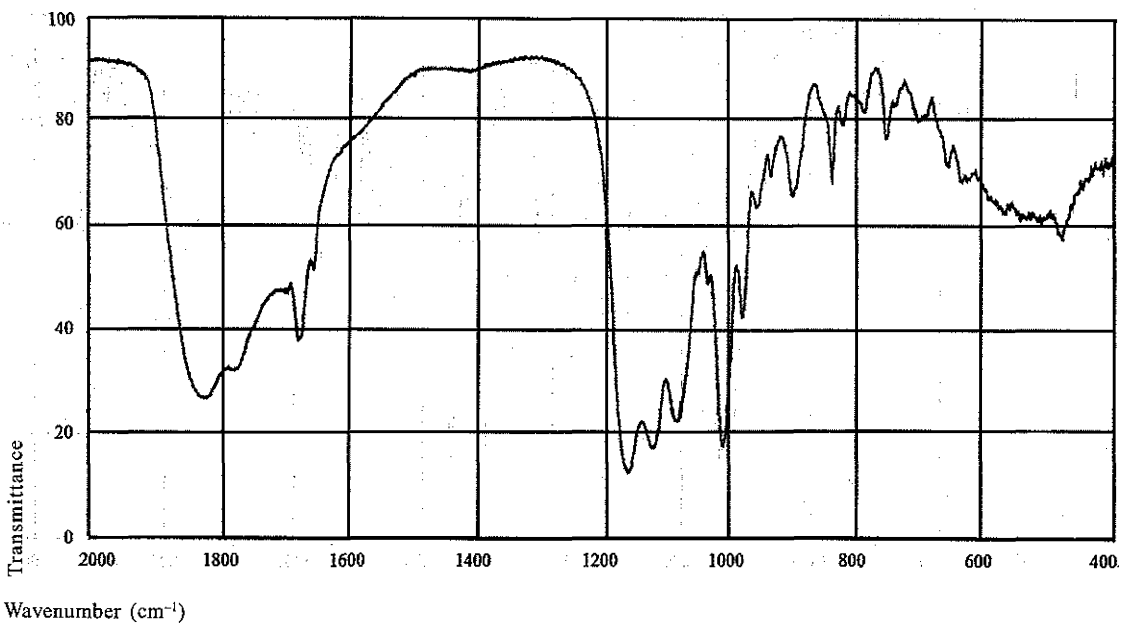
Ciclesonide *FTIR* Phase: KBr disc



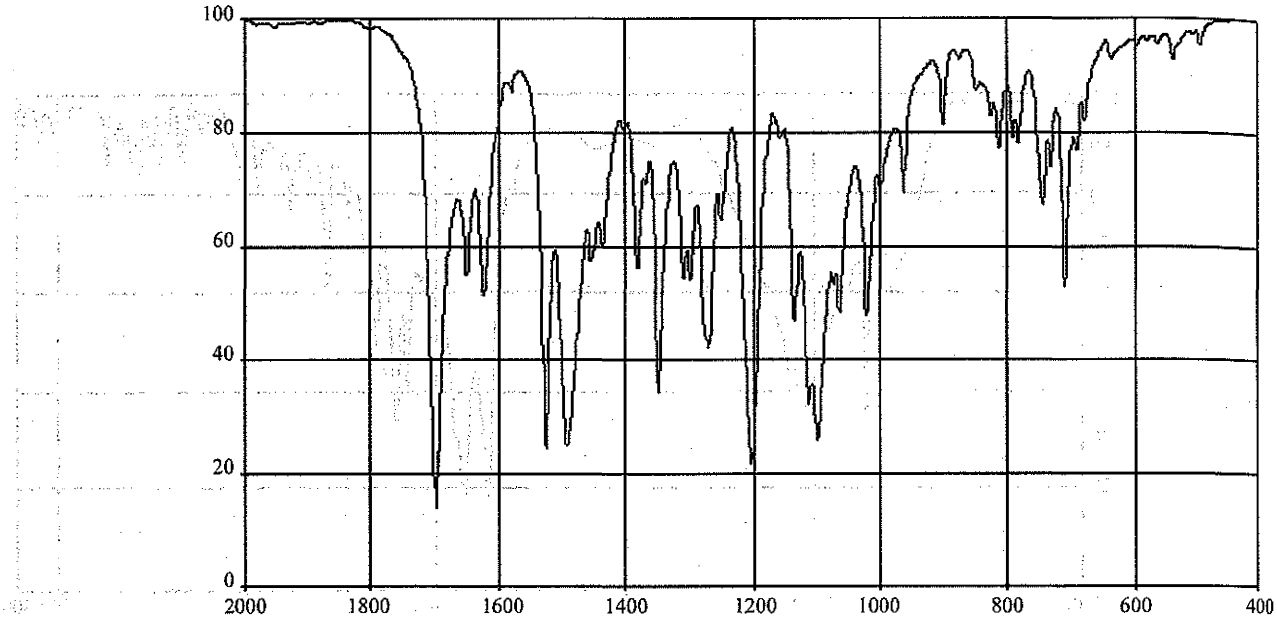
Cilastatin Ammonium *FTIR* Phase: KBr disc



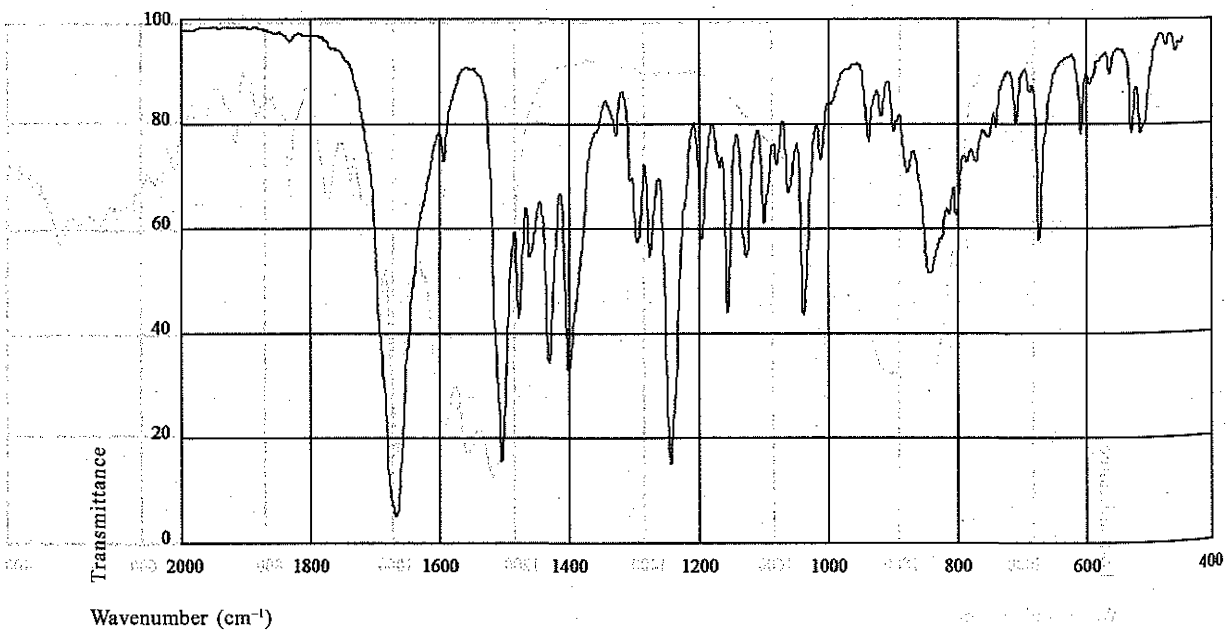
Cilastatin Sodium *FTIR* Phase: KBr disc



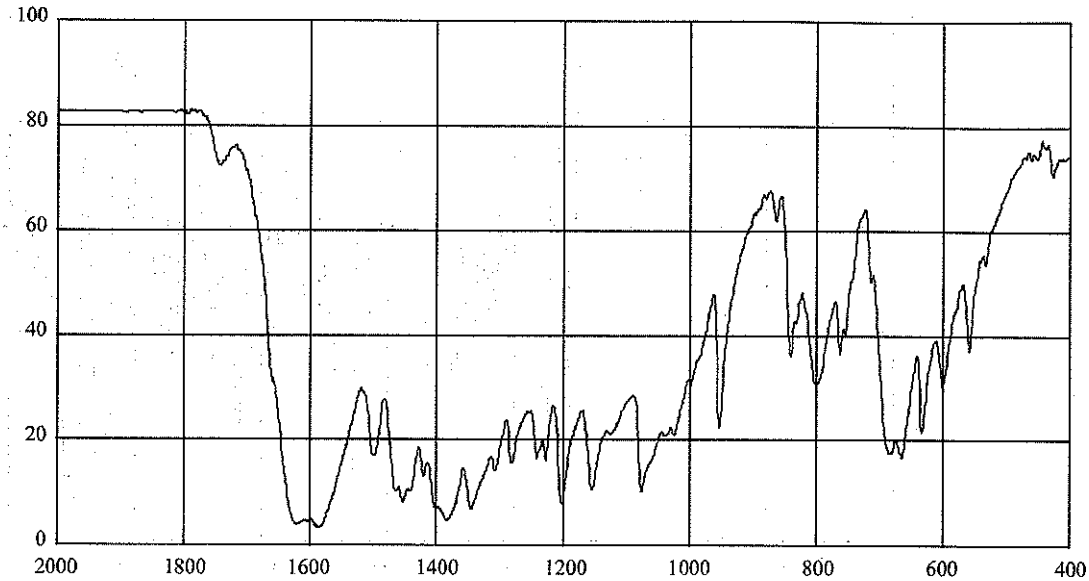
Cilnidipine *FTIR* Phase: KBr disc



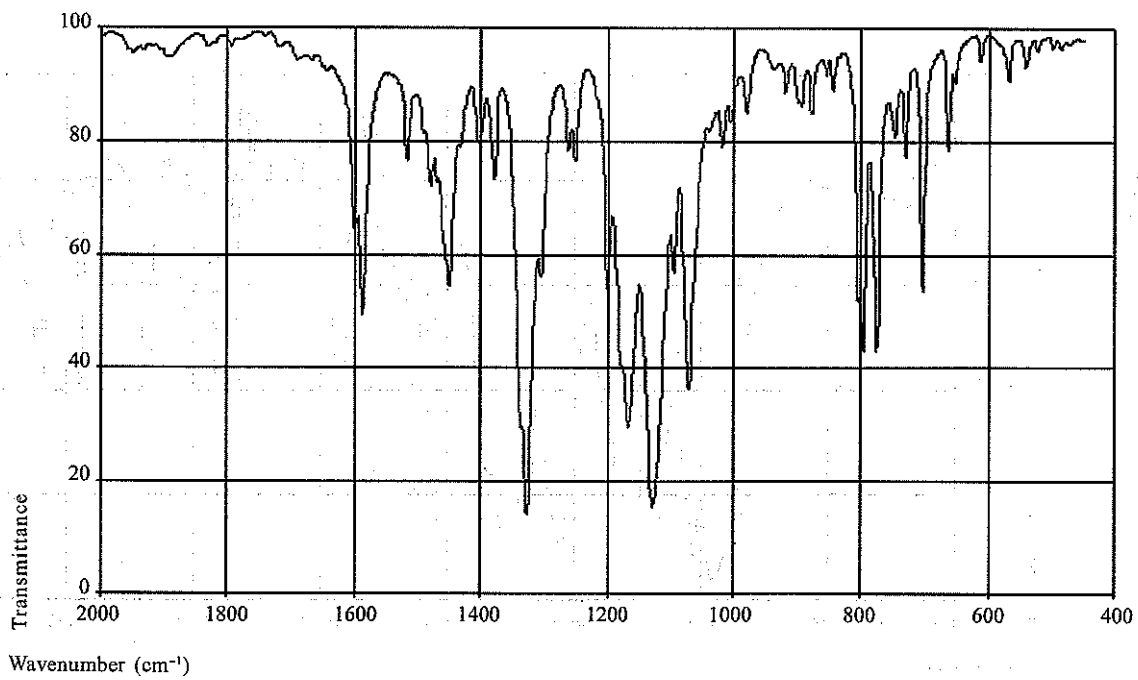
Cilostazol *FTIR* Phase: KBr disc



Cimetidine *FTIR* Phase: KBr disc



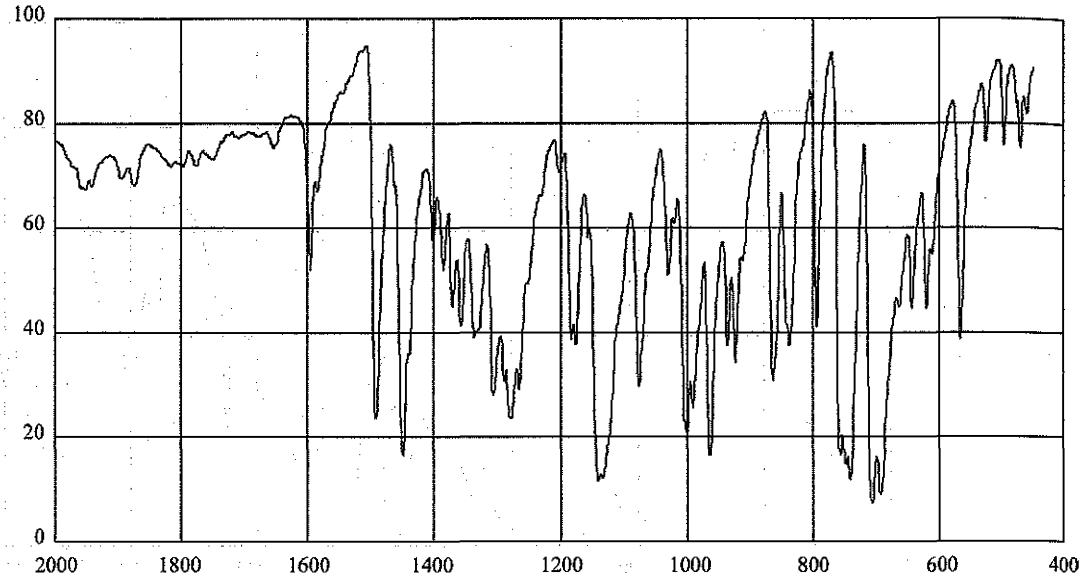
Cinacalcet Hydrochloride *FTIR* Phase: KBr disc



Cinnarazine

FTIR

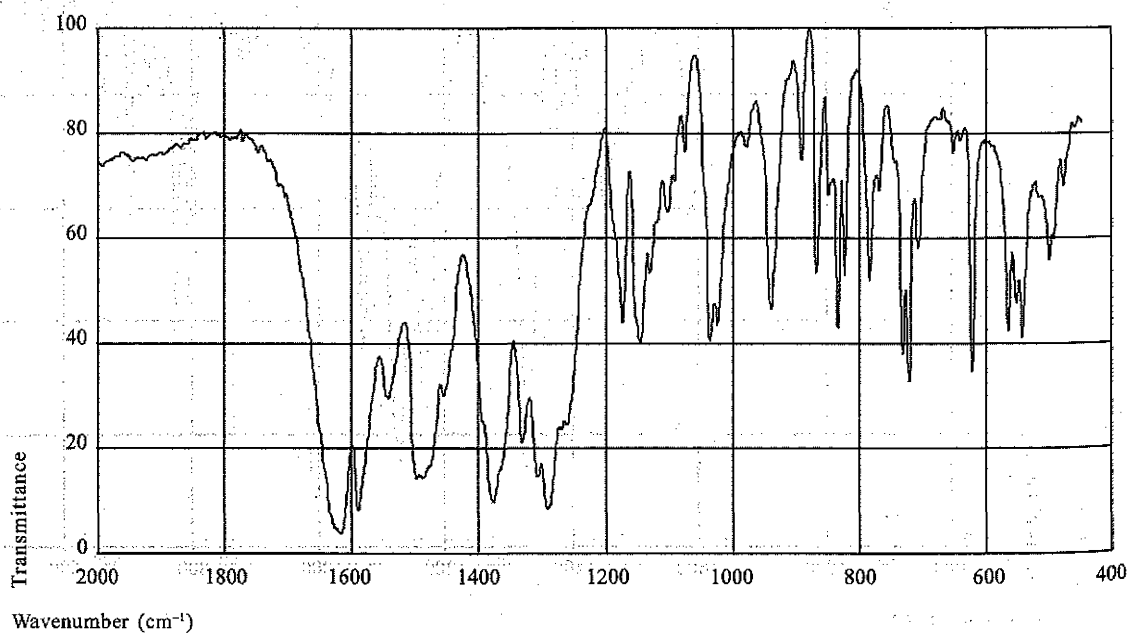
Phase: KBr disc



Ciprofloxacin

FTIR

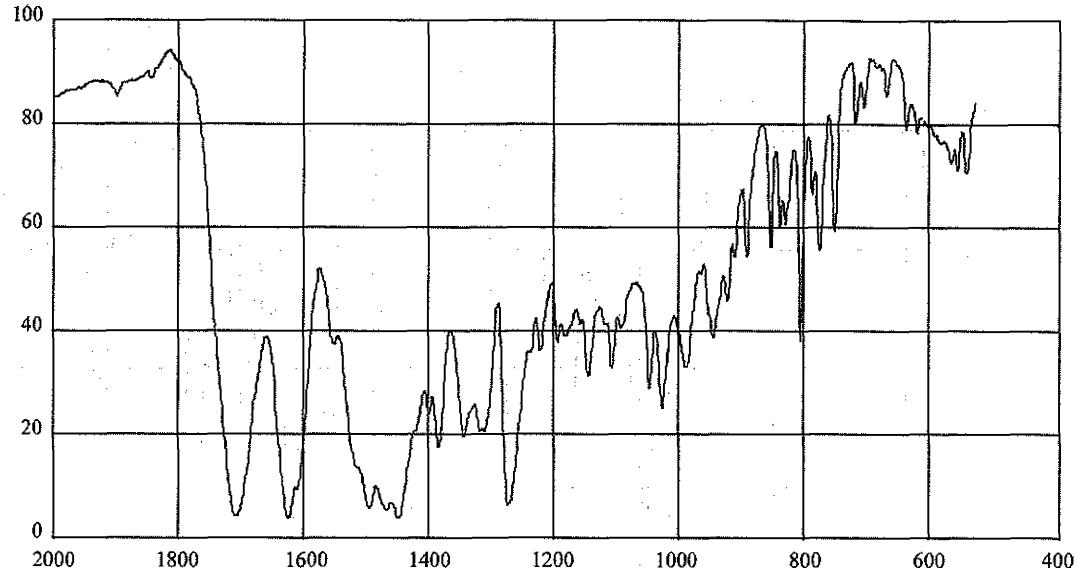
Phase: KBr disc



Ciprofloxacin Hydrochloride

FTIR

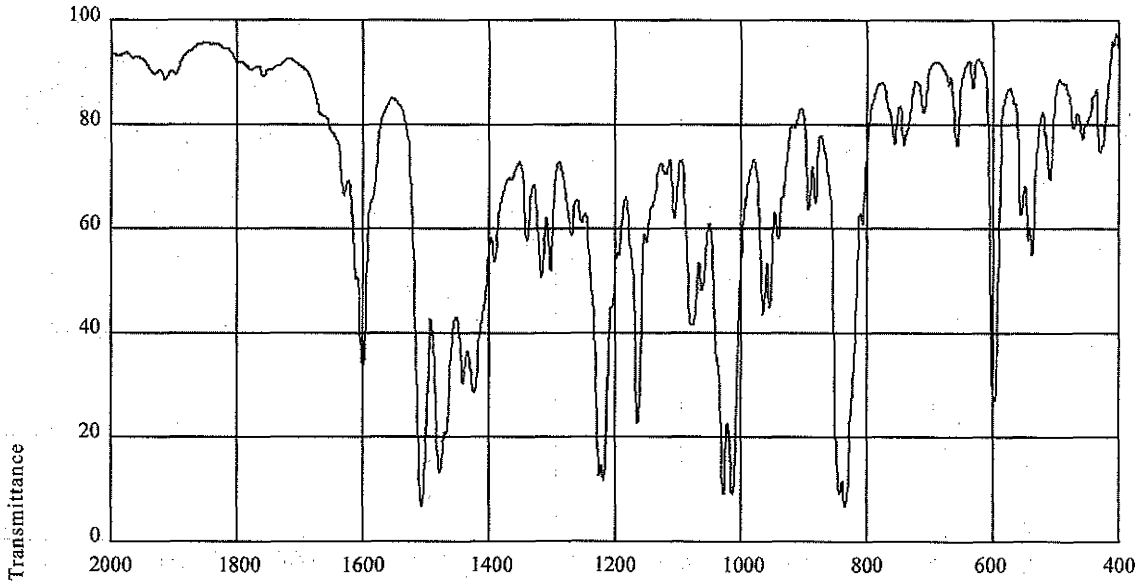
Phase: KBr disc



Citalopram Hydrobromide

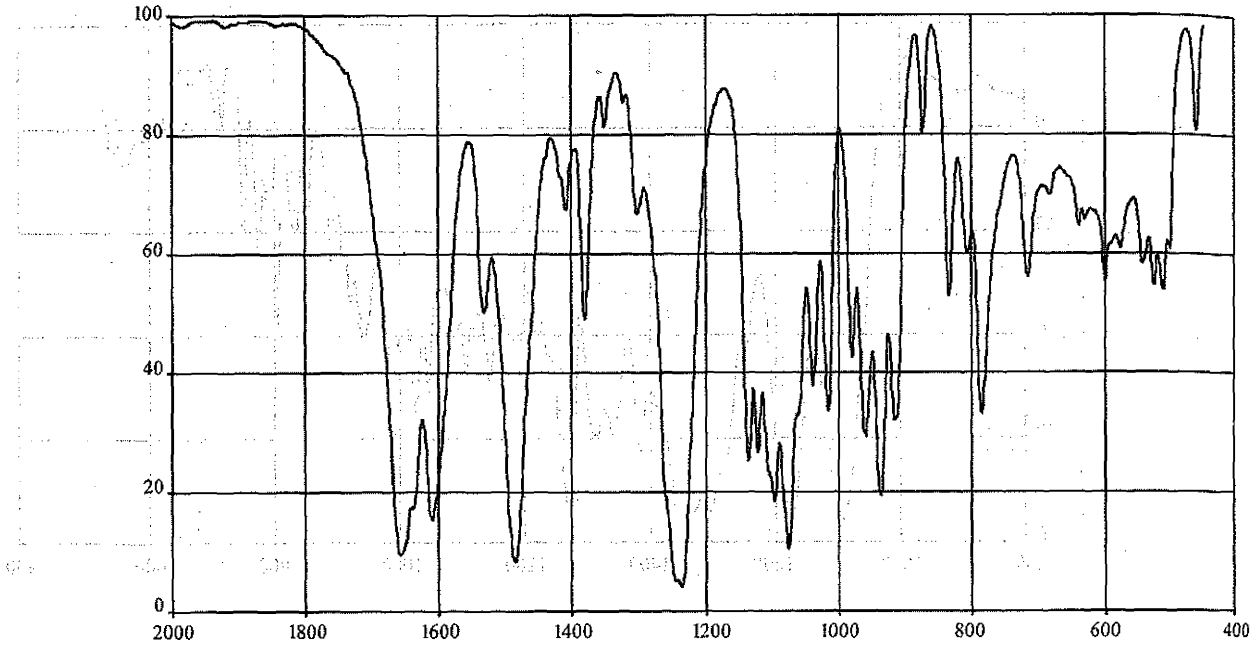
FTIR

Phase: KBr disc



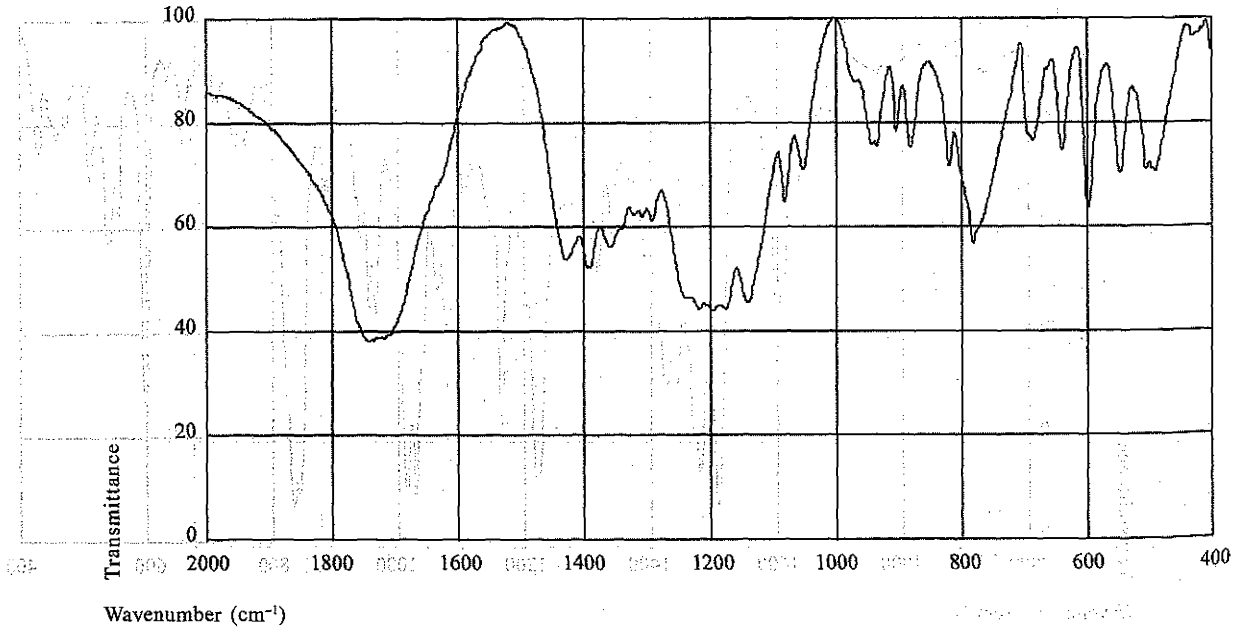
Citicoline Sodium

FTIR Phase: KBr disc



Citric Acid

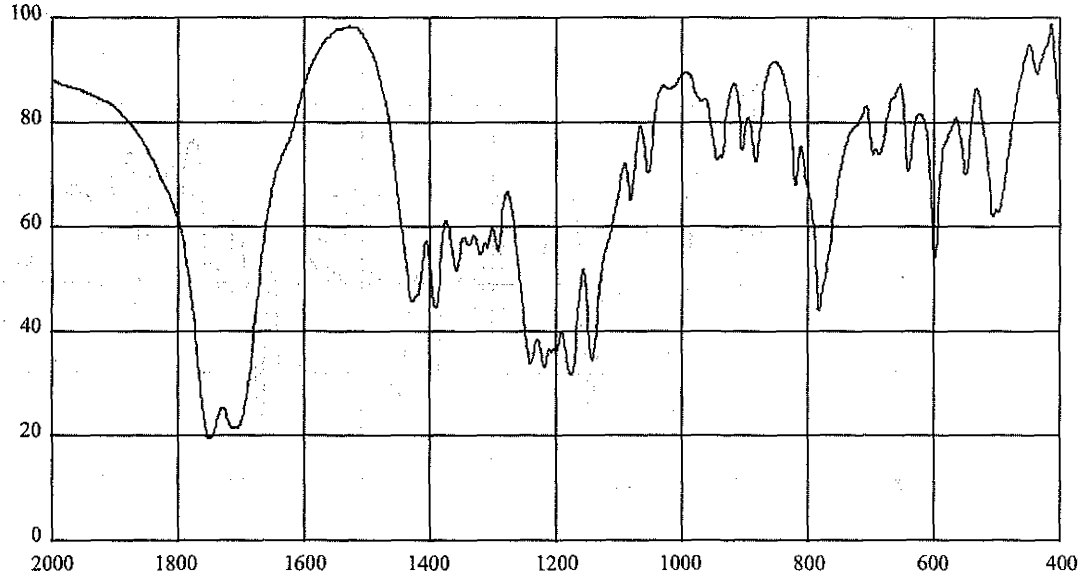
FTIR Phase: KBr disc



Citric Acid Monohydrate

FTIR

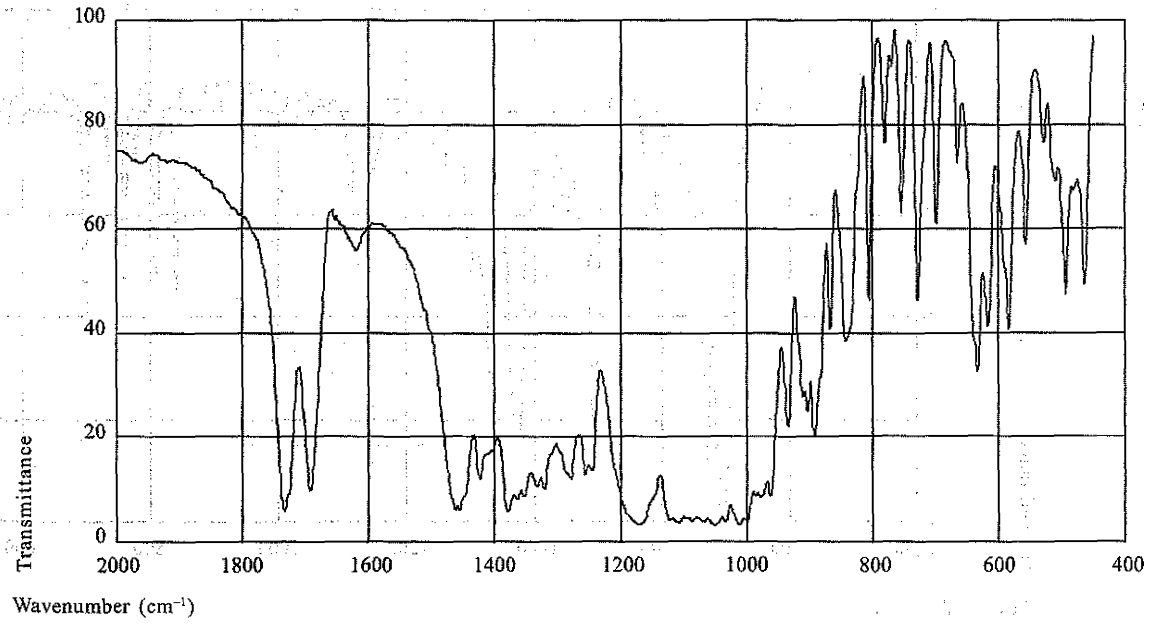
Phase: KBr disc



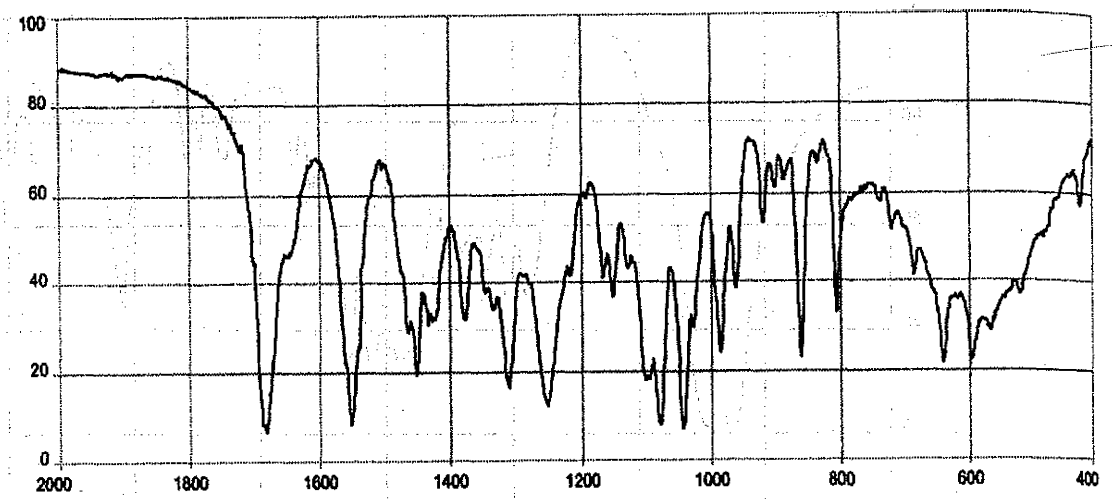
Clarithromycin

FTIR

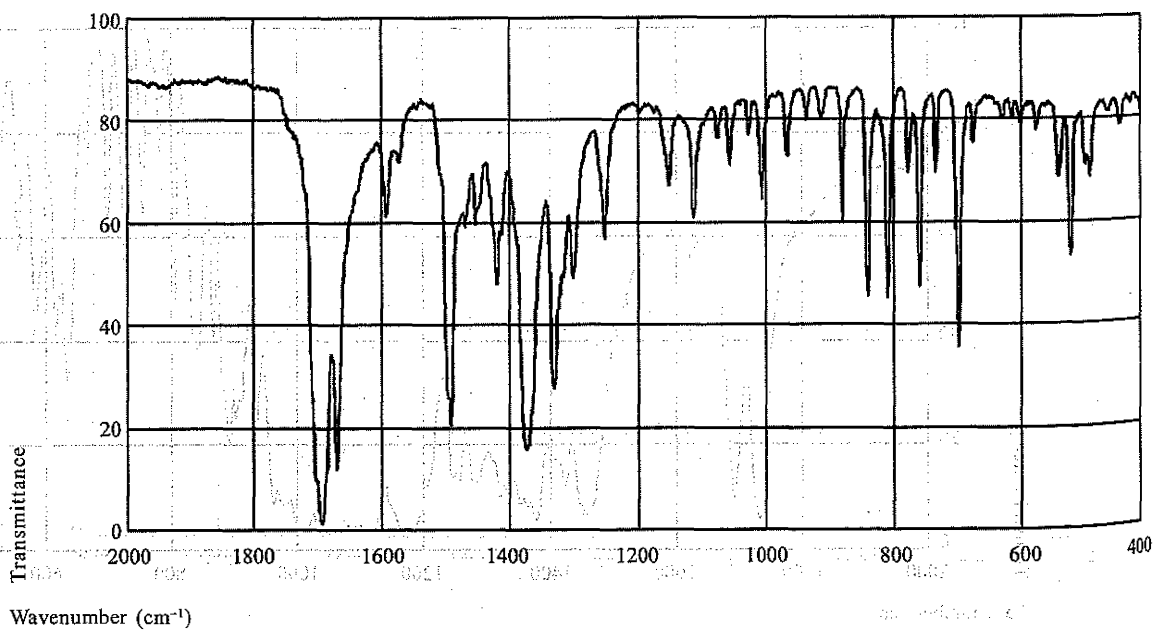
Phase: KBr disc



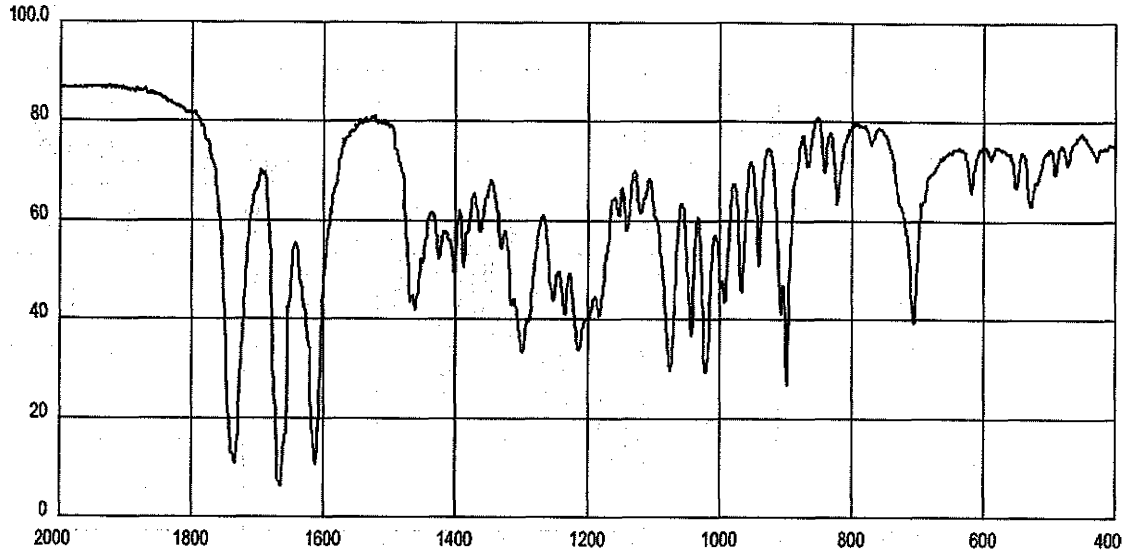
Clindamycin Hydrochloride *Dispersive IR* Phase: KCl disc



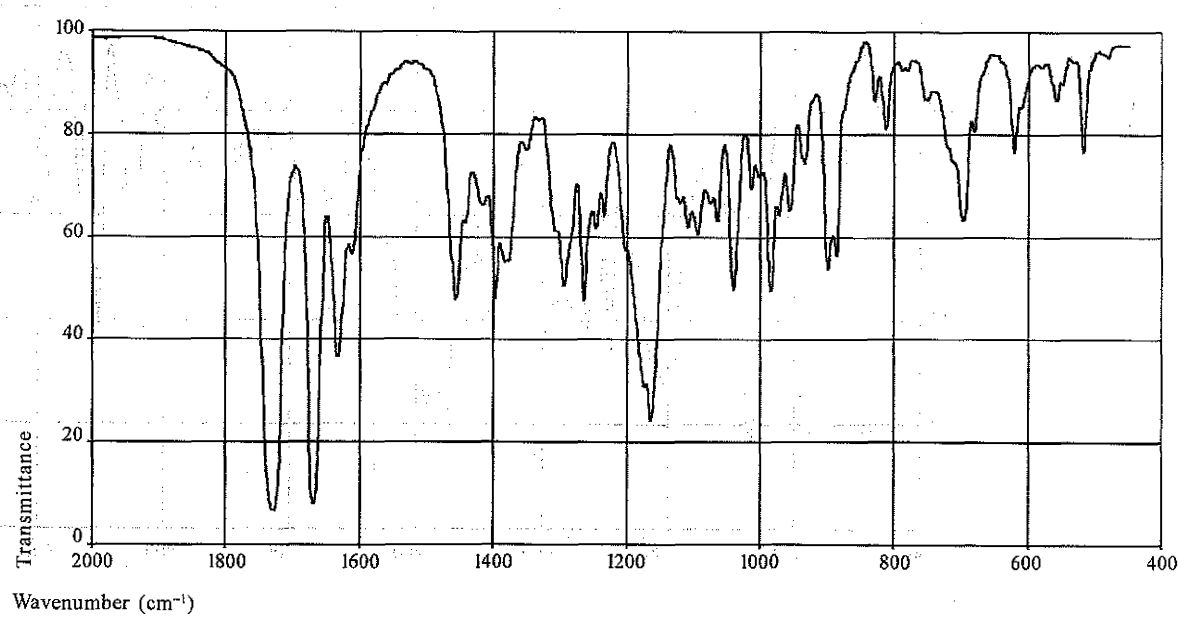
Clobazam *FTIR* Phase: KBr disc



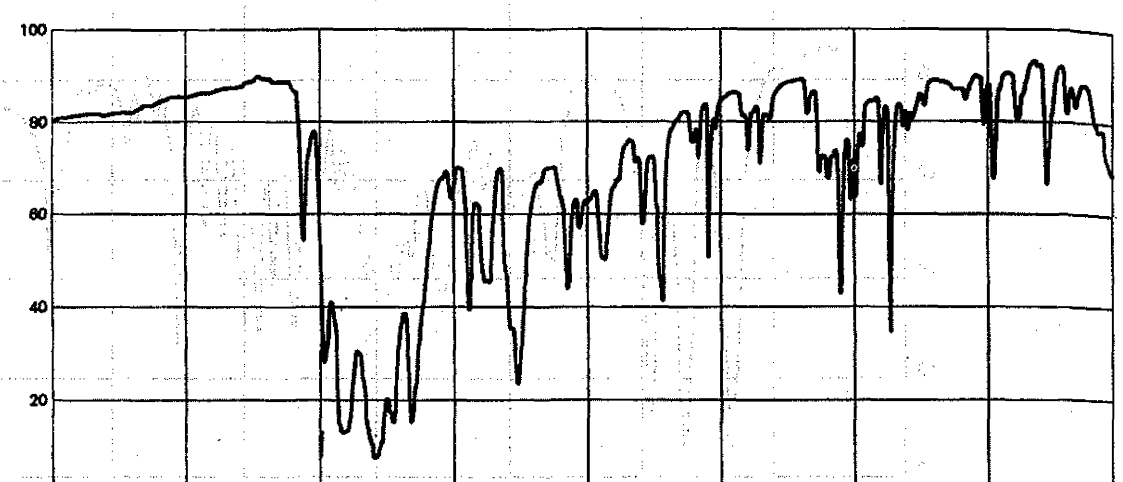
Clobetasol Propionate *FTIR* Phase: KBr disc



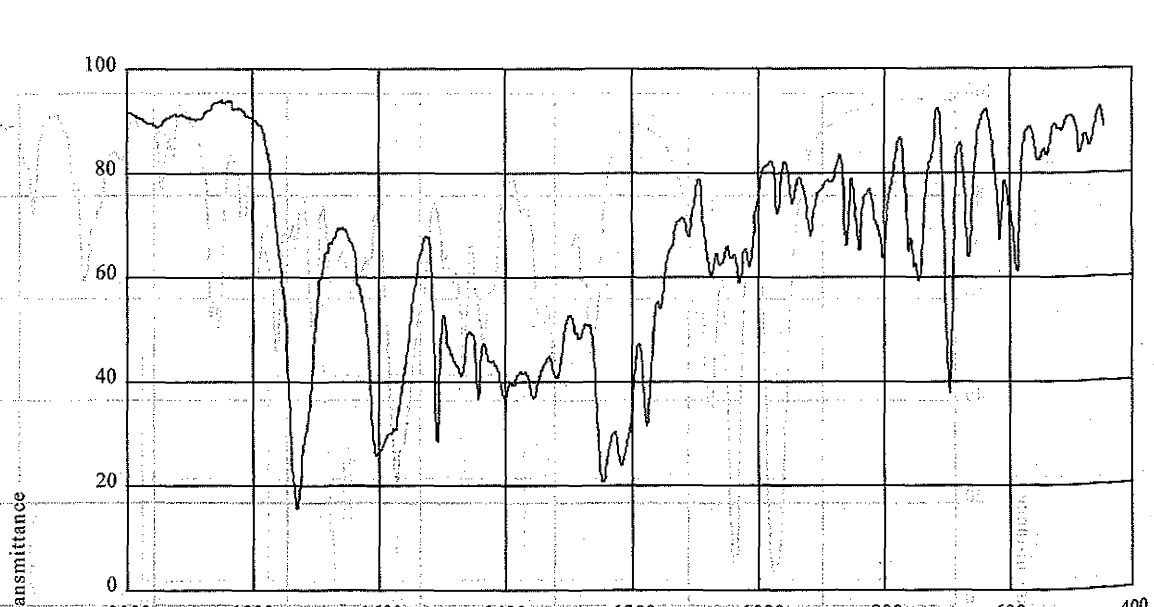
Clobetasone Butyrate *FTIR* Phase: KBr disc



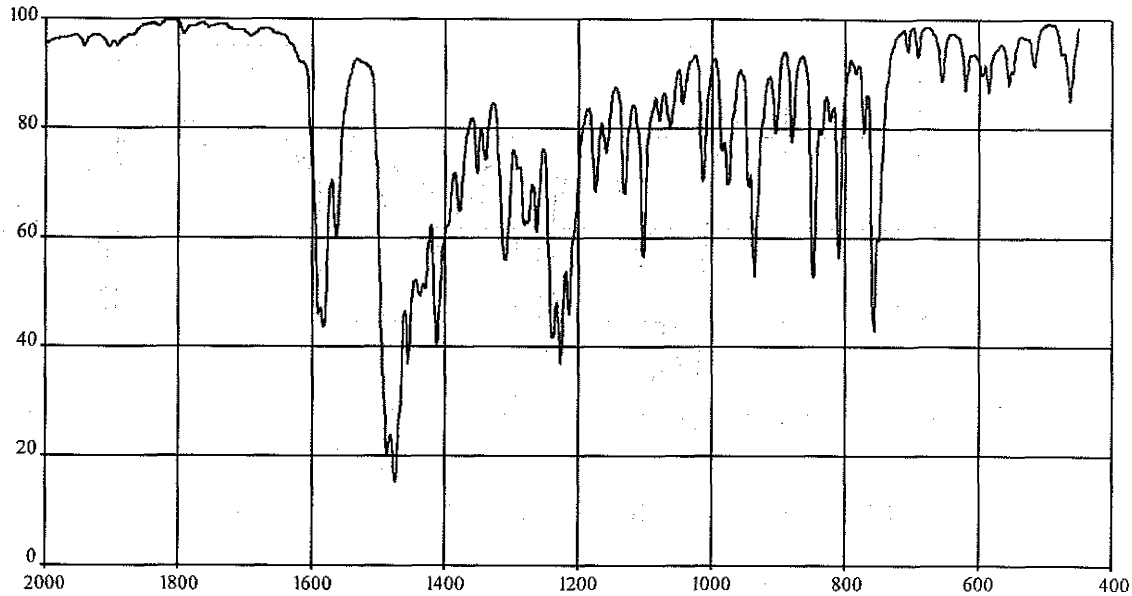
Clofazimine *Dispersive IR* Phase: Liquid paraffin mull



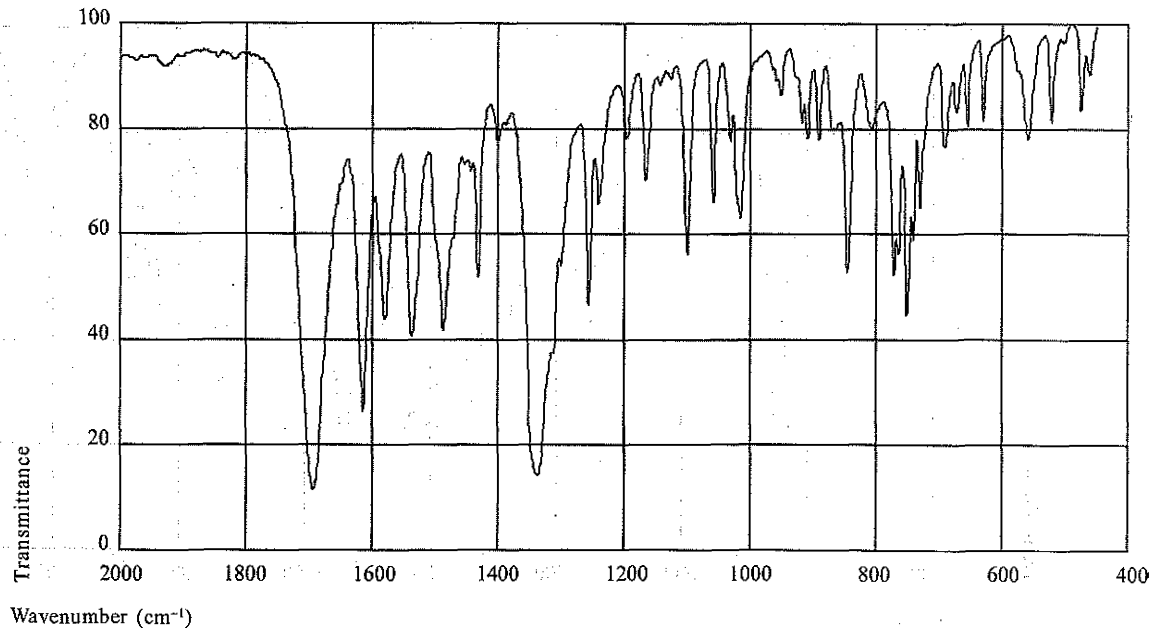
Clomifene Citrate *FTIR* Phase: KBr disc



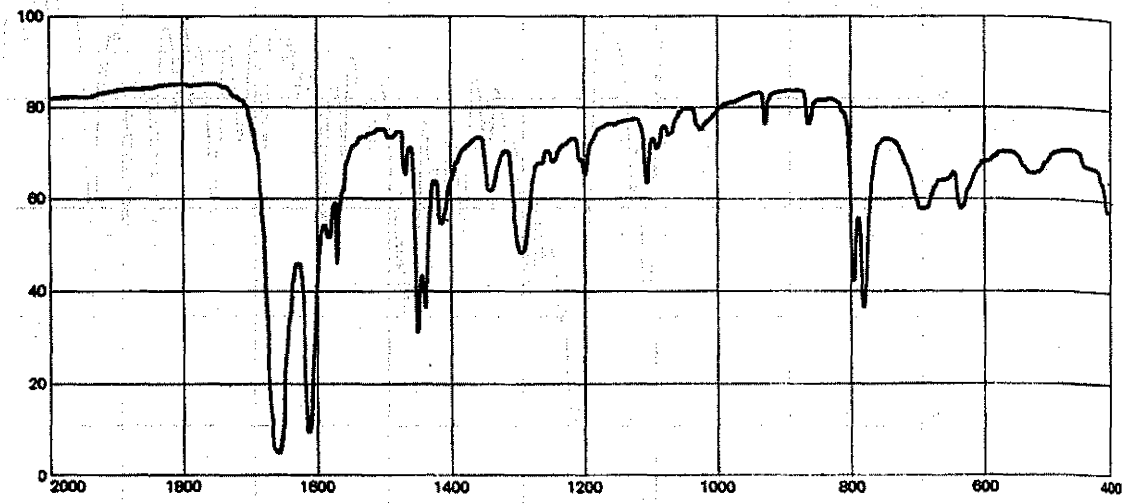
Clomipramine Hydrochloride *FTIR* Phase: KBr disc



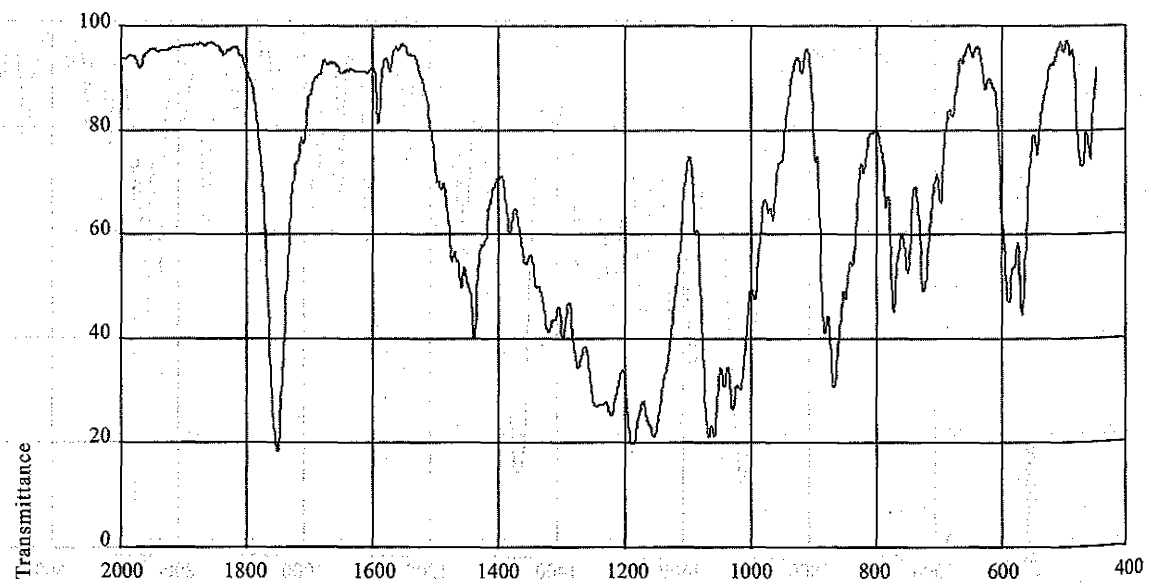
Clonazepam *FTIR* Phase: KBr disc



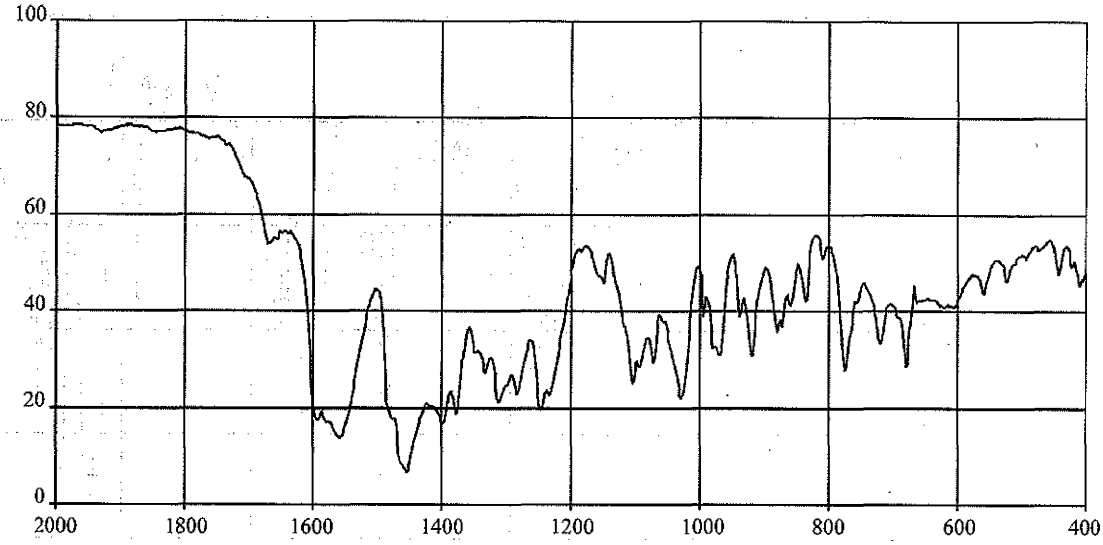
Clonidine Hydrochloride *Dispersive IR* Phase: KBr disc



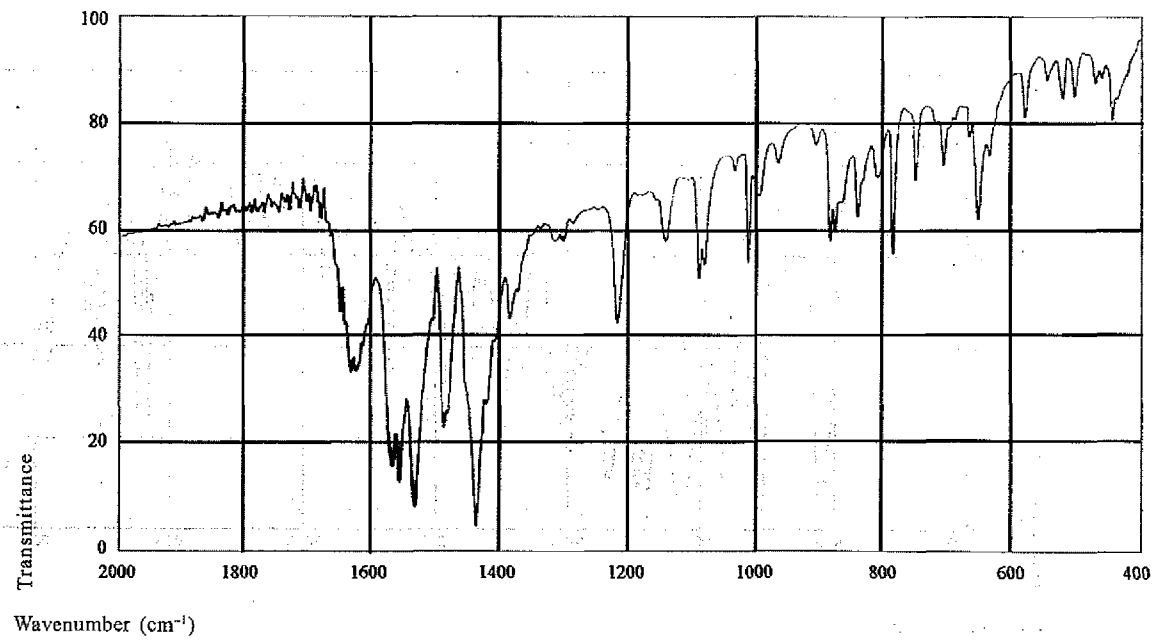
Clopidogrel Bisulphate *FTIR* Phase: KBr disc



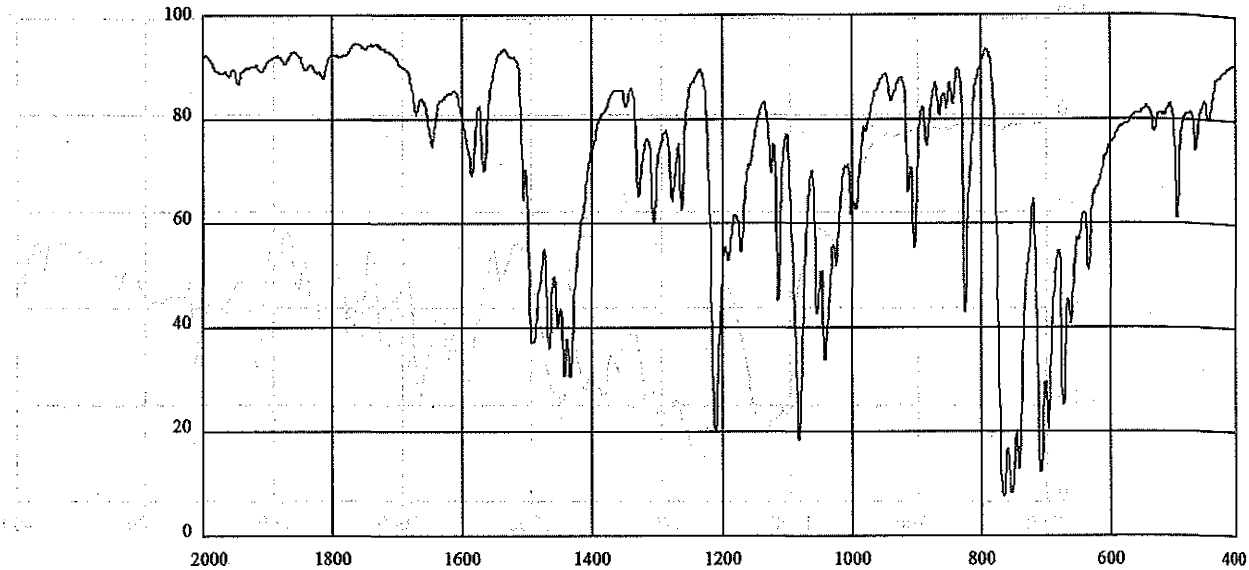
Cloprostenol Sodium *FTIR* Phase: KBr disc



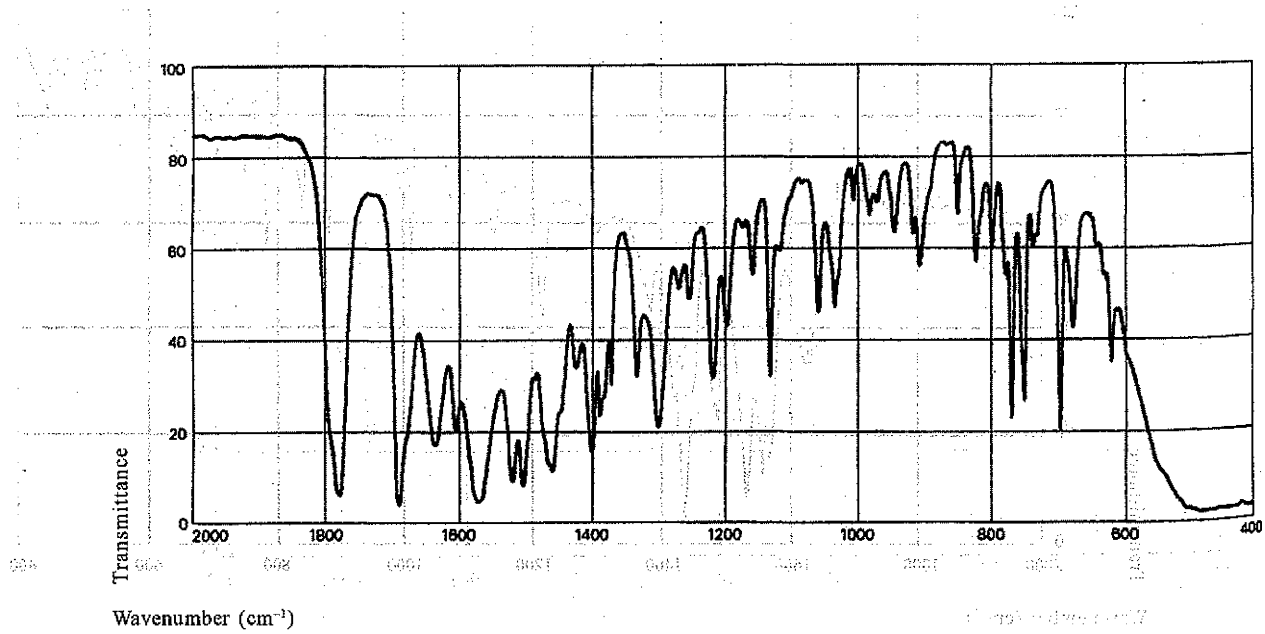
Closental Sodium *FTIR* Phase: KBr disc



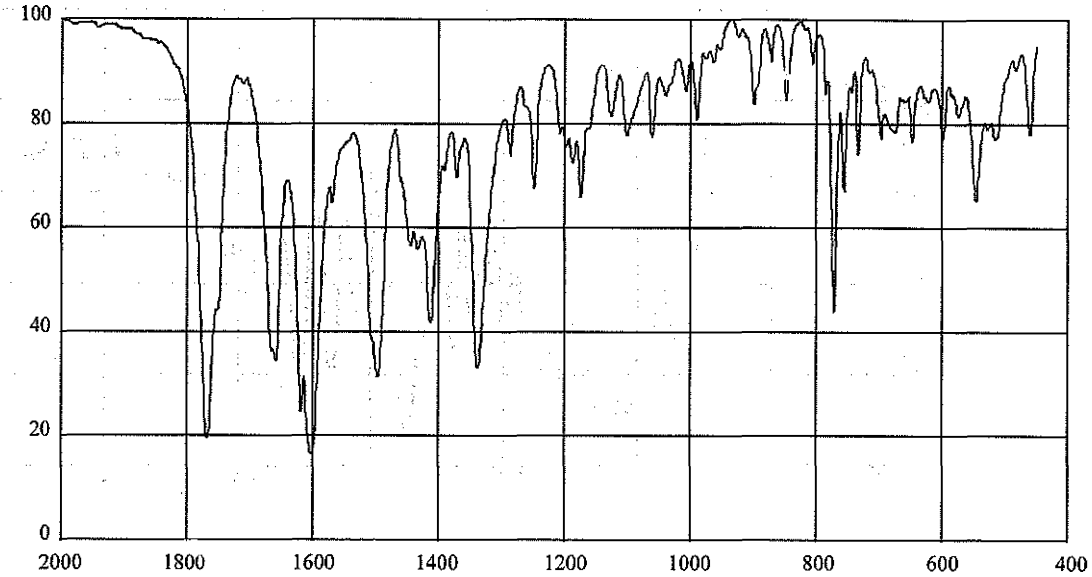
Clotrimazole *FTIR* Phase: KBr disc



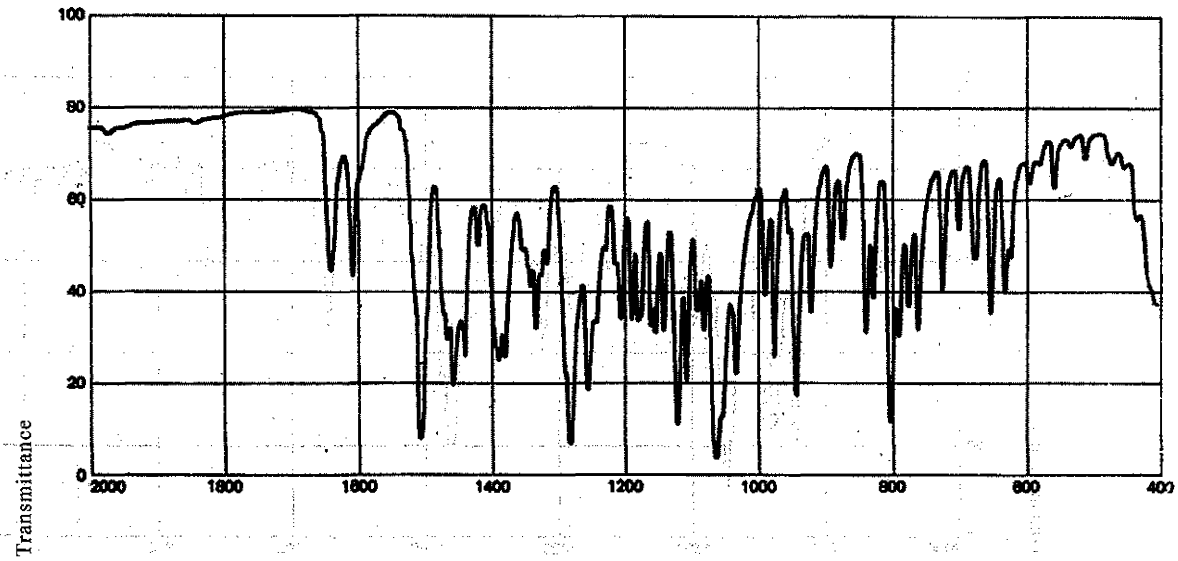
Cloxacillin Benzathine *Dispersive IR* Phase: Liquid paraffin mull



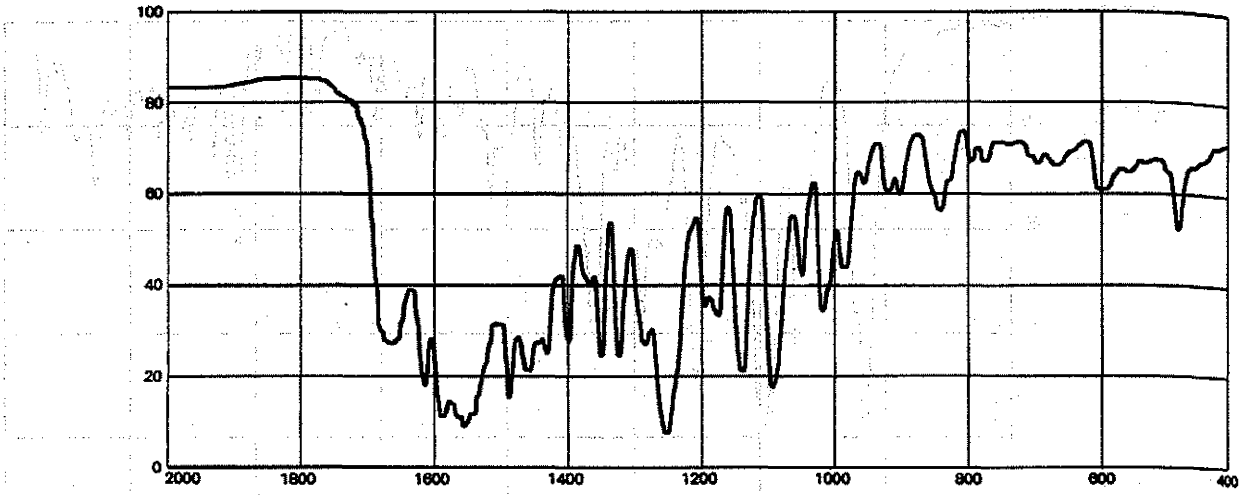
Cloxacillin Sodium *FTIR* Phase: KBr disc



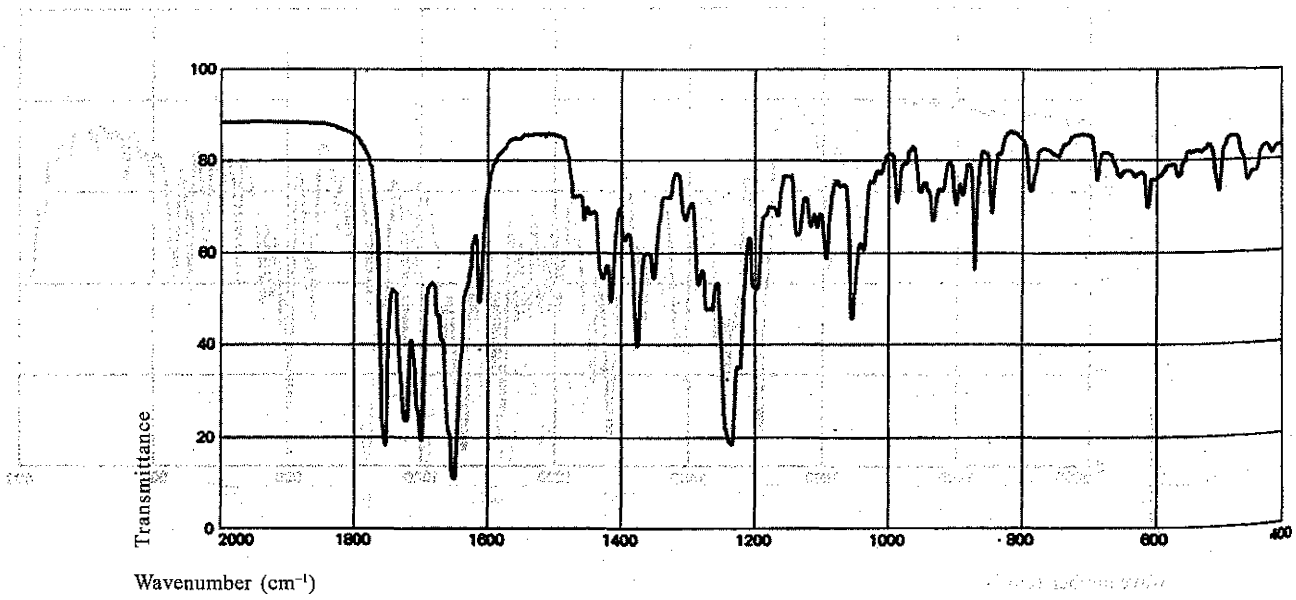
Codeine *Dispersive IR* Phase: KBr disc



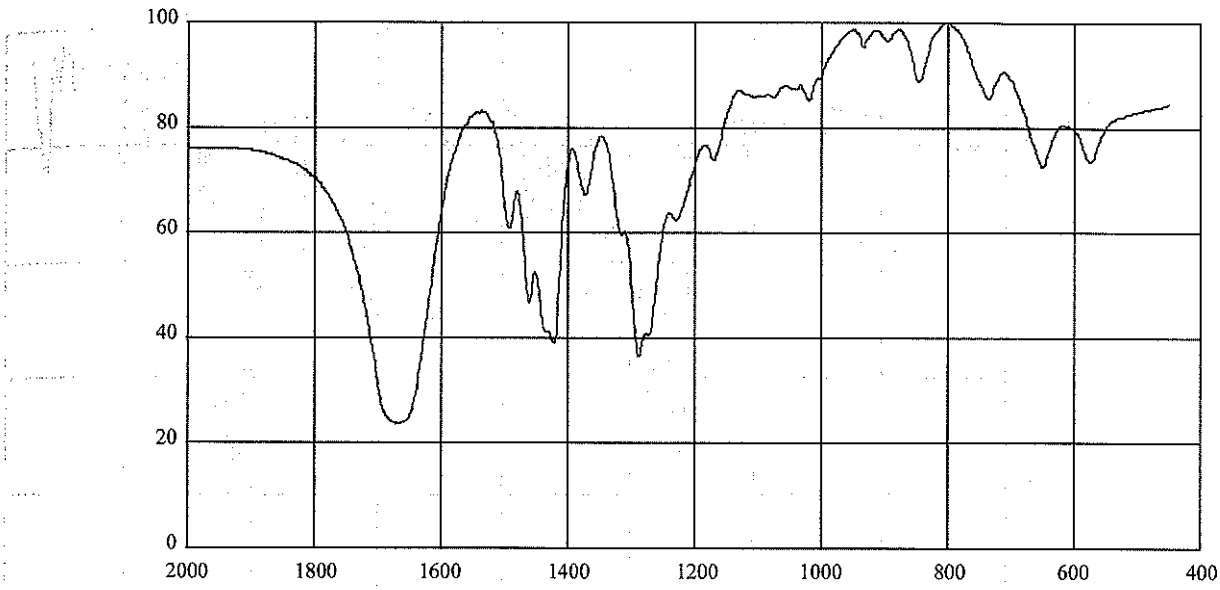
Colchicine *Dispersive IR* Phase: KBr disc



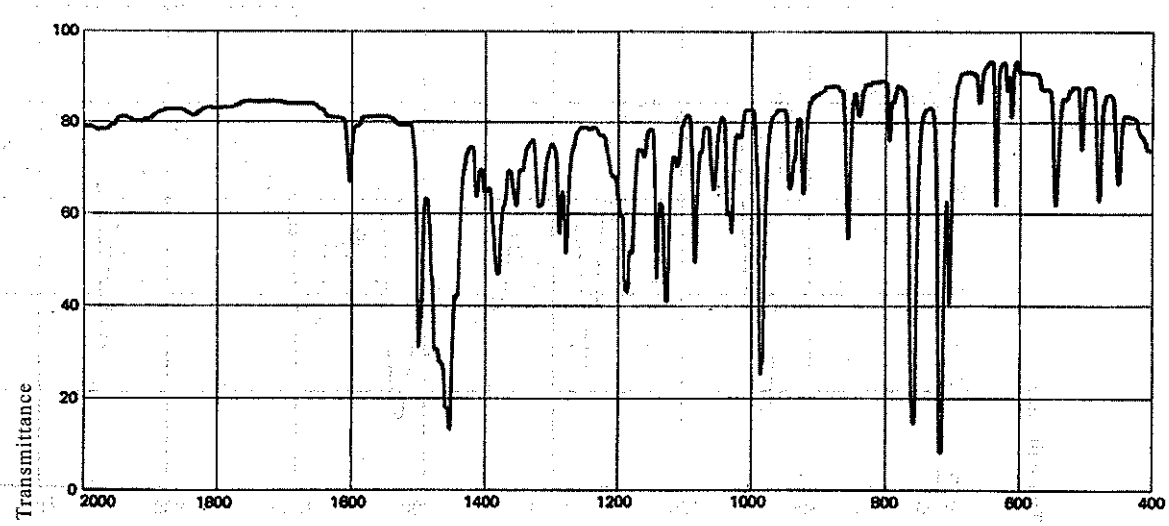
Cortisone Acetate *Dispersive IR* Phase: KBr disc



Crospovidone *FTIR* Phase: KBr disc



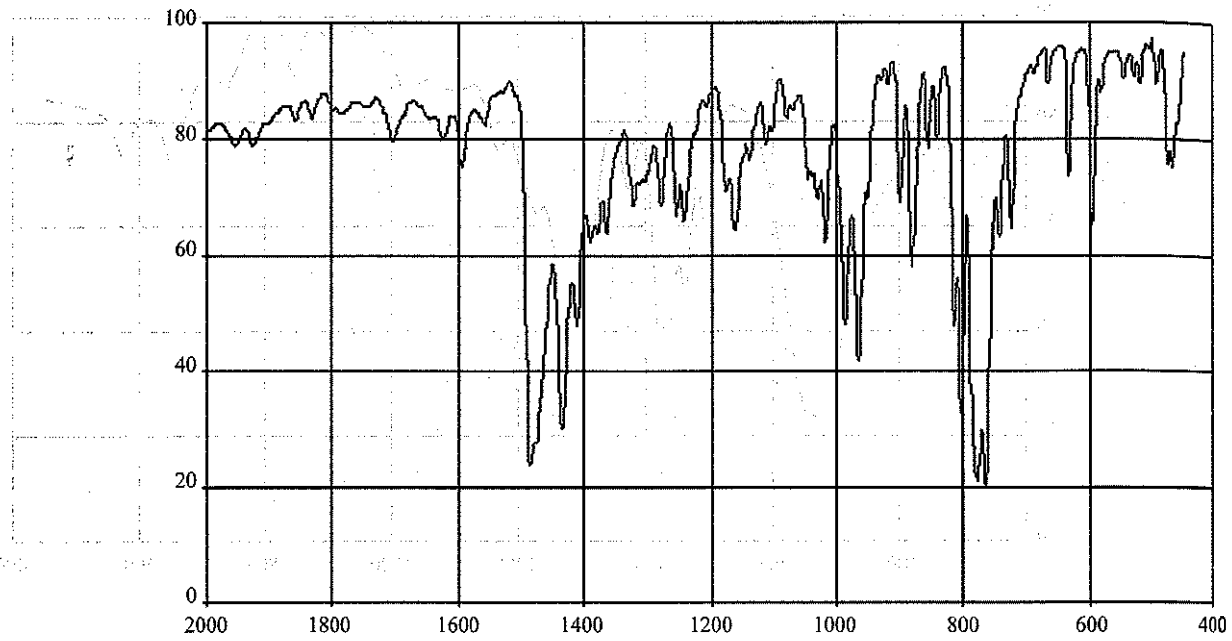
Cyclizine Hydrochloride *Dispersive IR* Phase: Liquid paraffin mull



Cyclobenzaprine Hydrochloride

FTIR

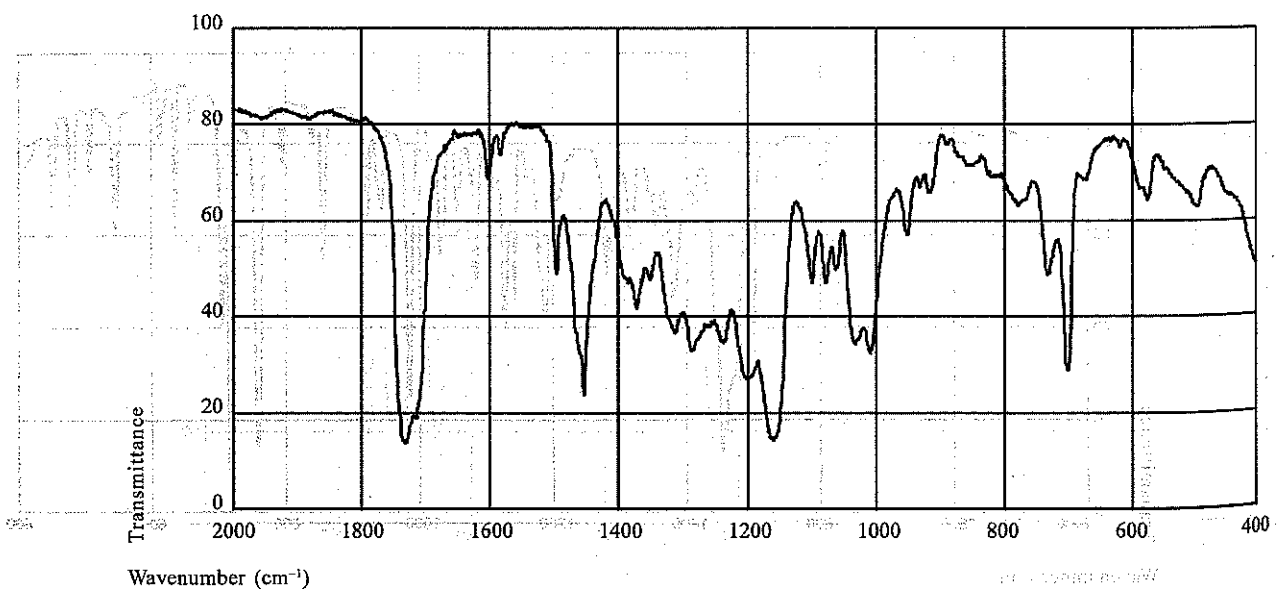
Phase: KBr disc



Cyclopentolate

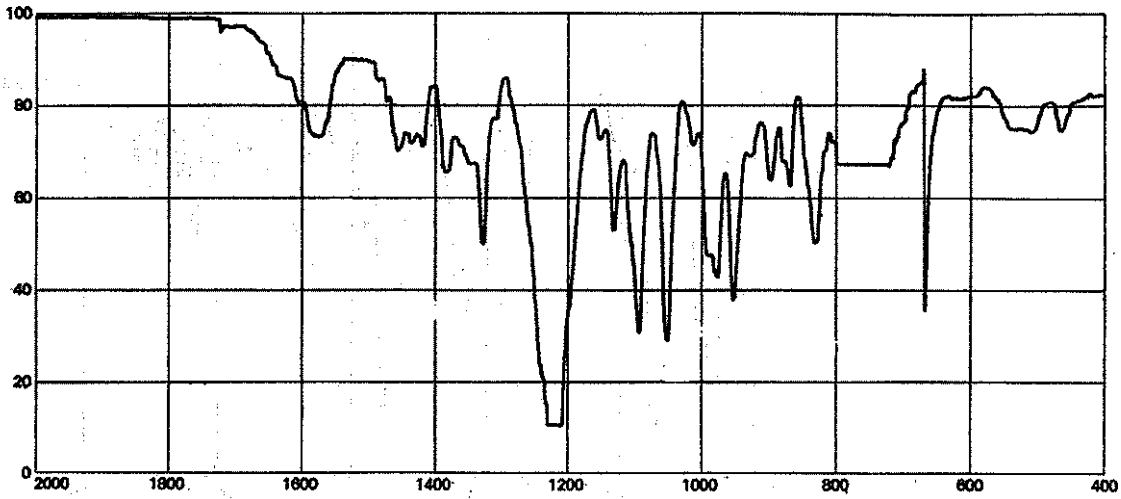
FTIR

Phase: KBr disc



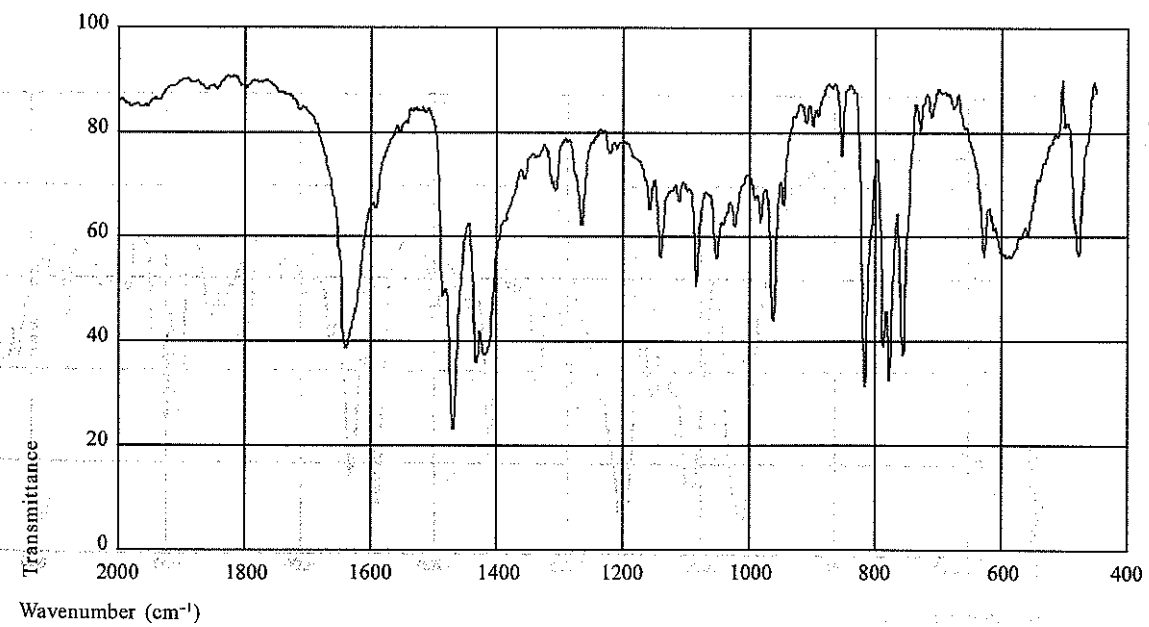
Cyclophosphamide

Dispersive IR Phase: 10 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm

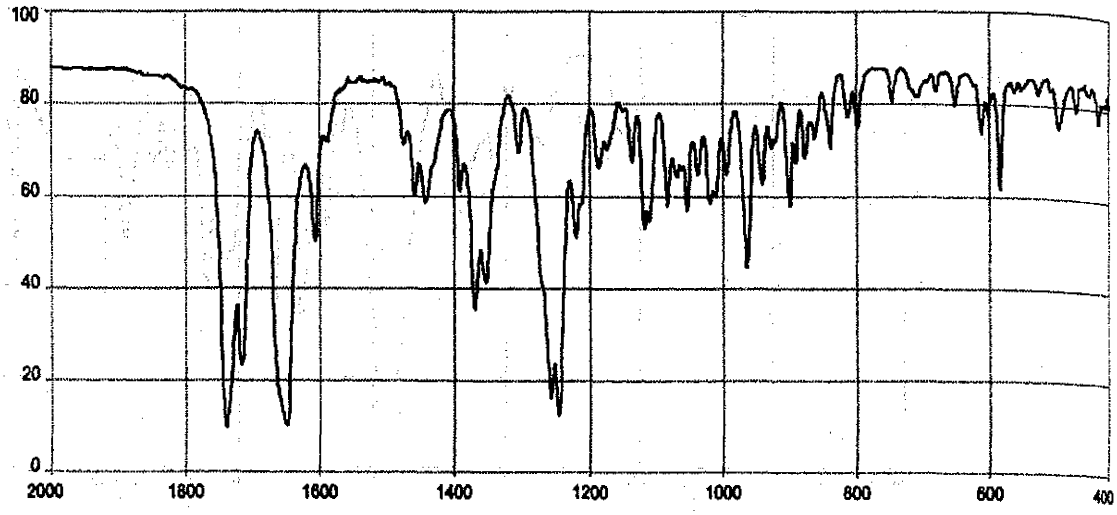


Cyproheptadine

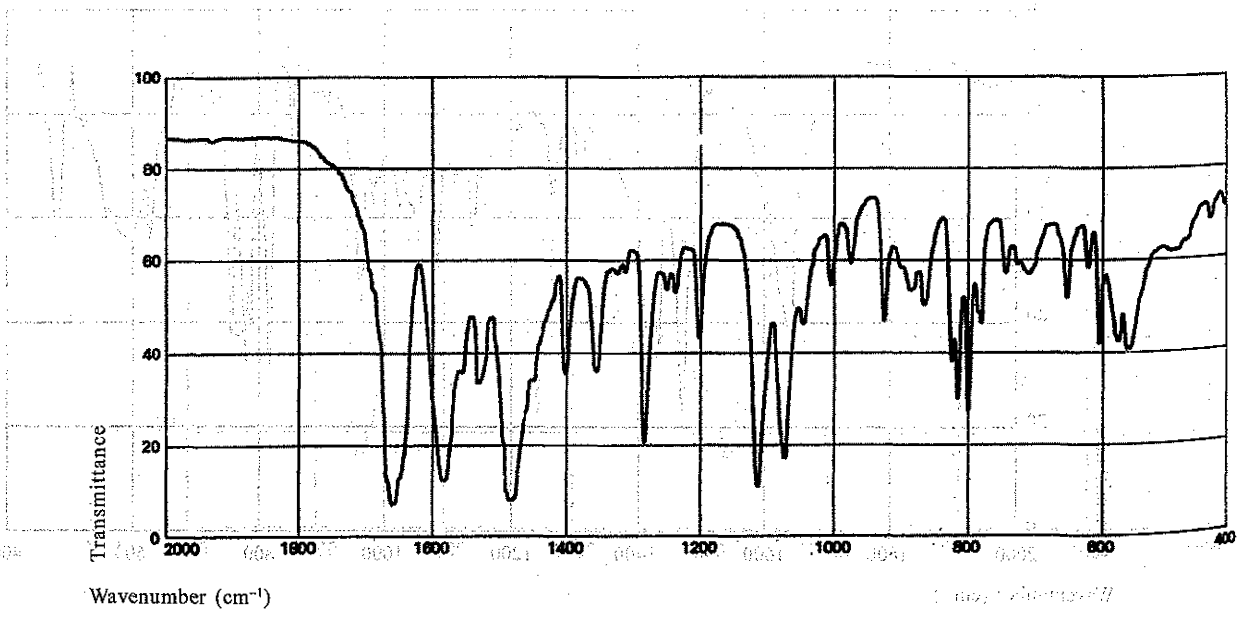
FTIR Phase: KBr disc



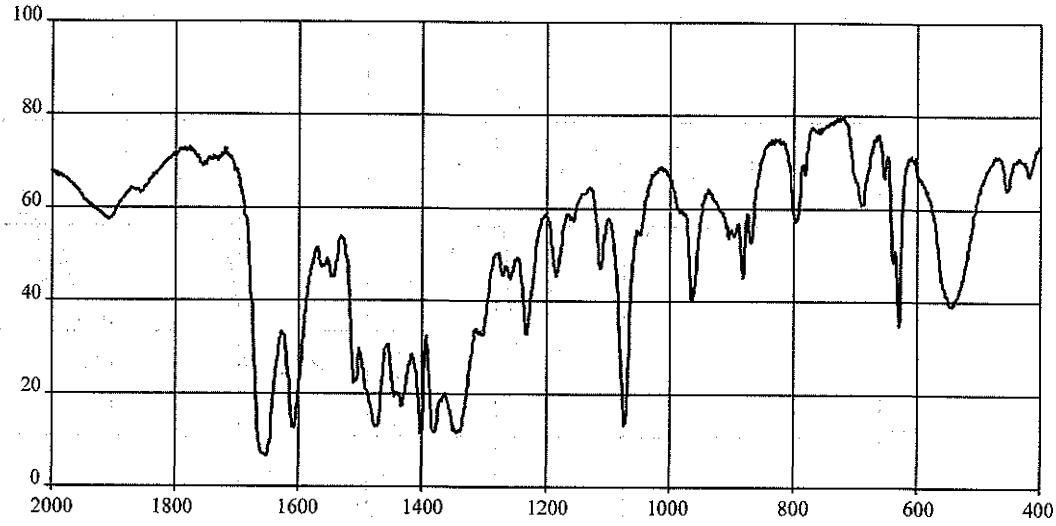
Cyproterone Acetate *Dispersive IR* Phase: KBr disc



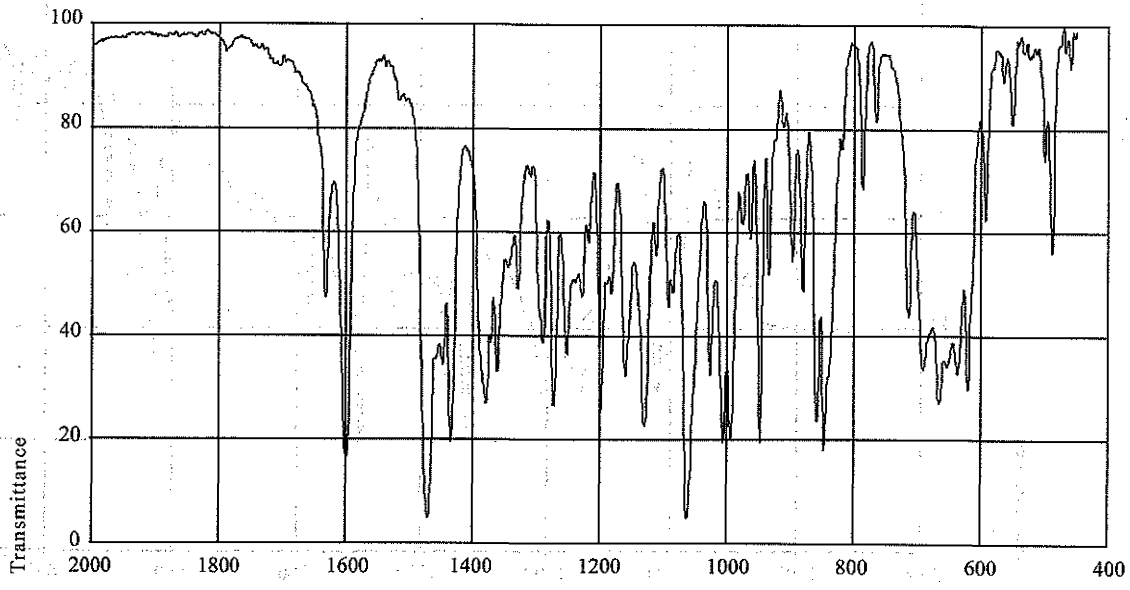
Cytarabine *Dispersive IR* Phase: KBr disc



Dacarbazine *FTIR* Phase: KBr disc



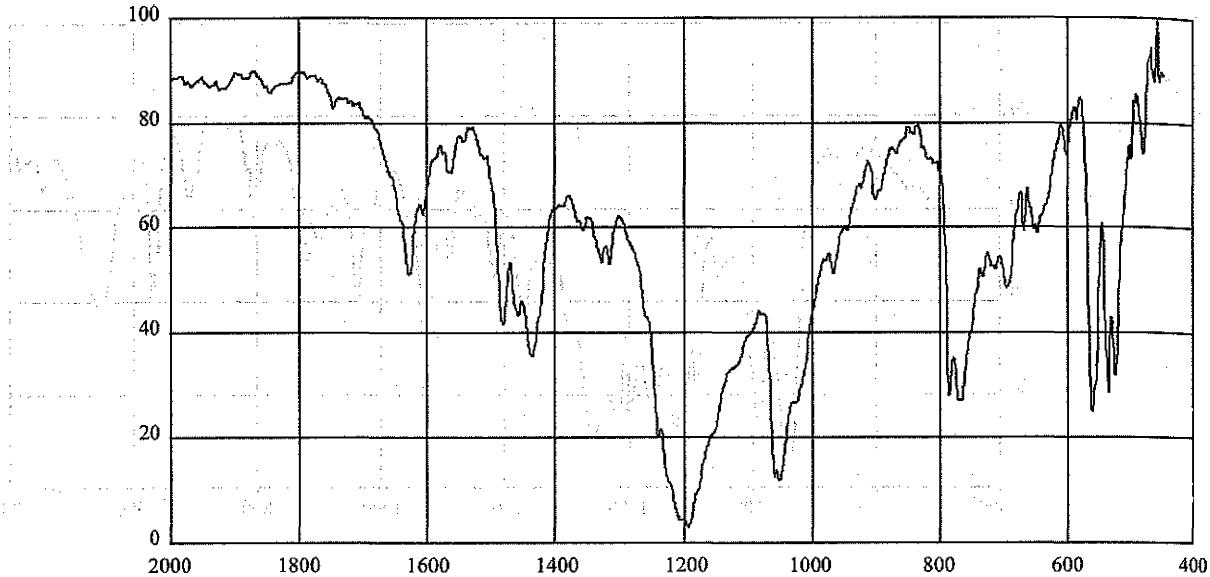
Danazol *FTIR* Phase: KBr disc



Dapoxetine Hydrochloride

FTIR

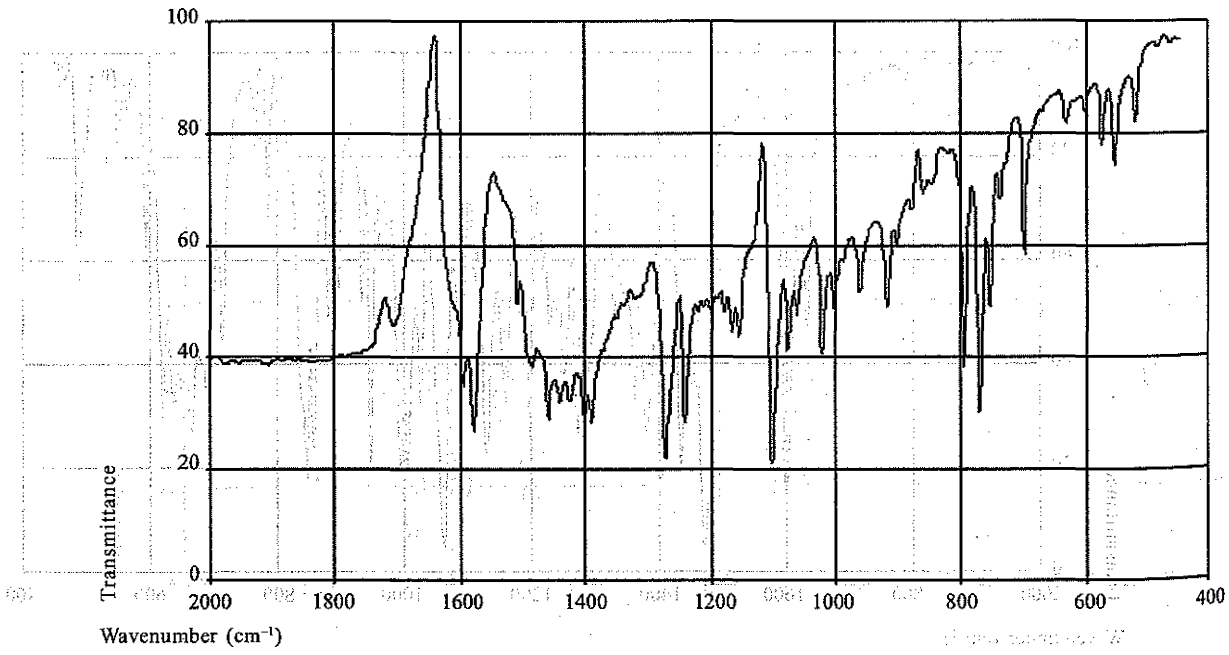
Phase: KBr disc



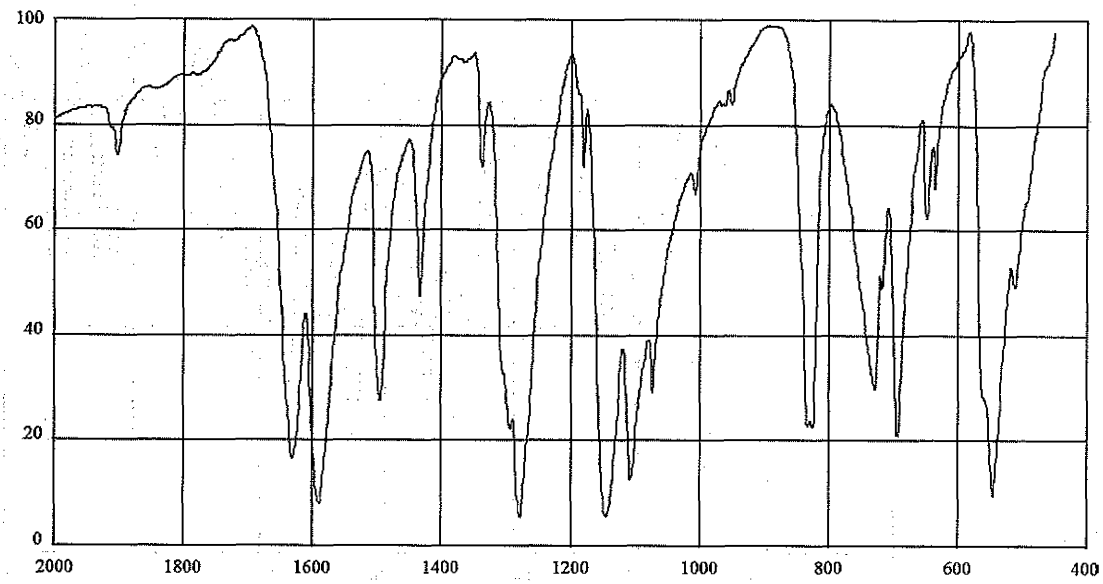
S-Dapoxetine Hydrochloride

FTIR

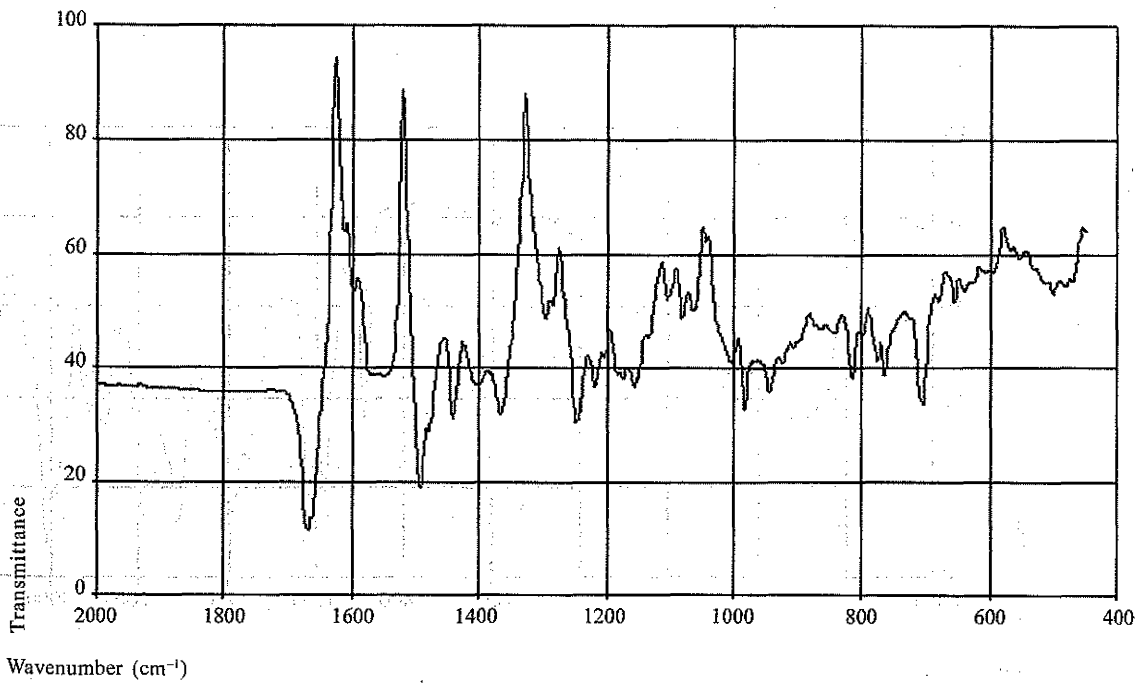
Phase: KBr disc



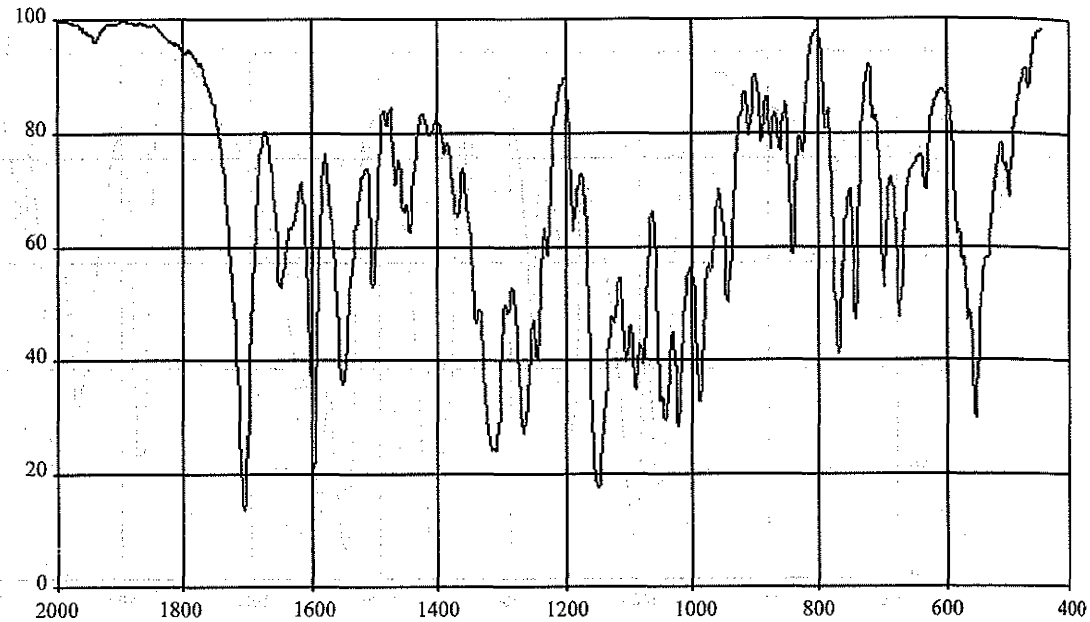
Dapsone *FTIR* Phase: KBr disc



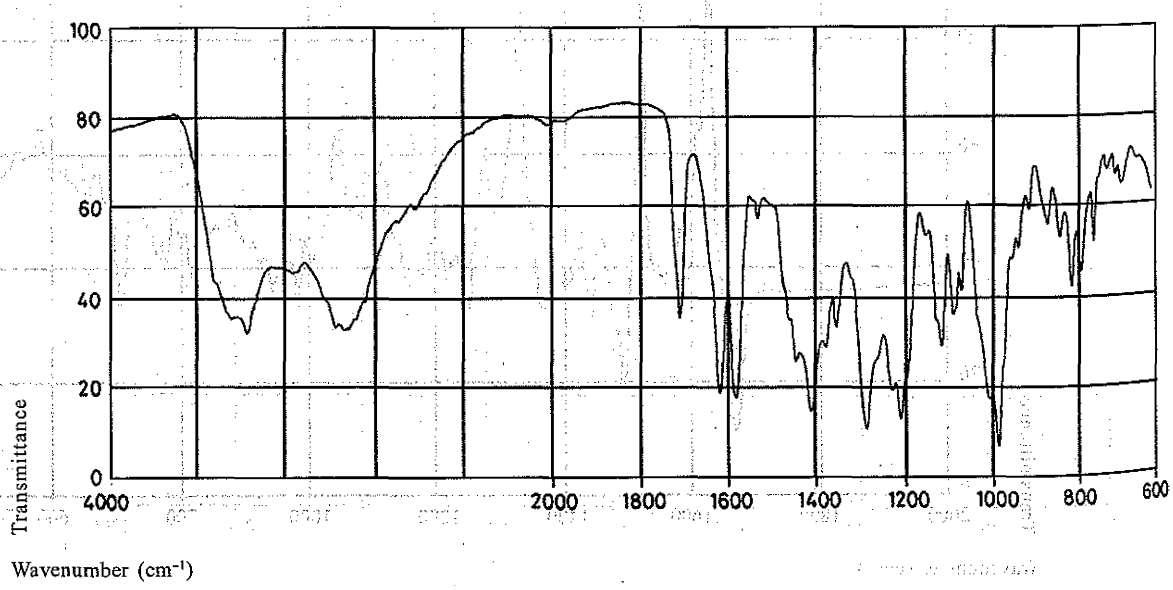
Darifenacin Hydrobromide *FTIR* Phase: KBr disc



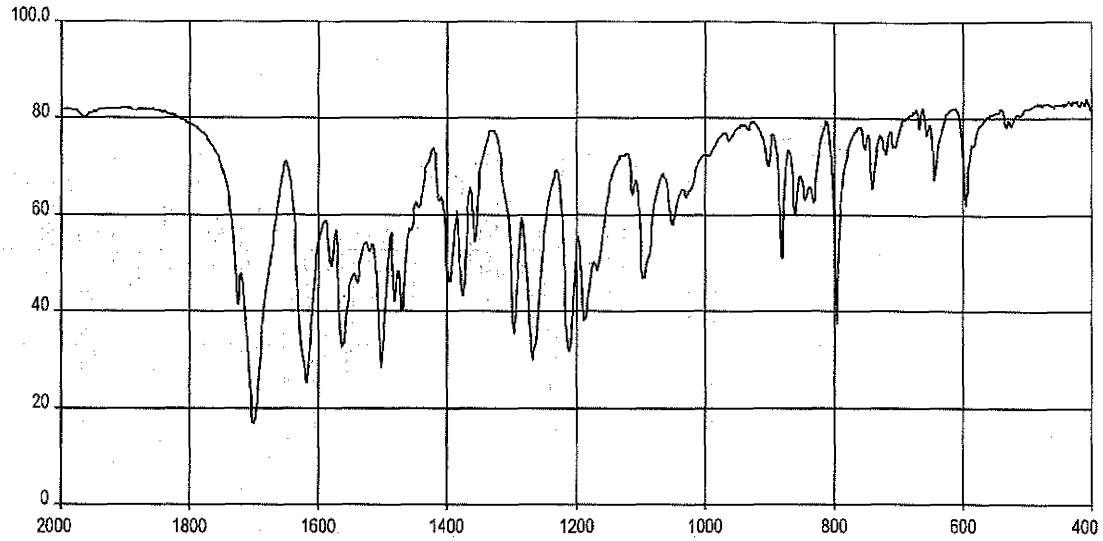
Darunavir Ethanolate *FTIR* Phase: KBr disc



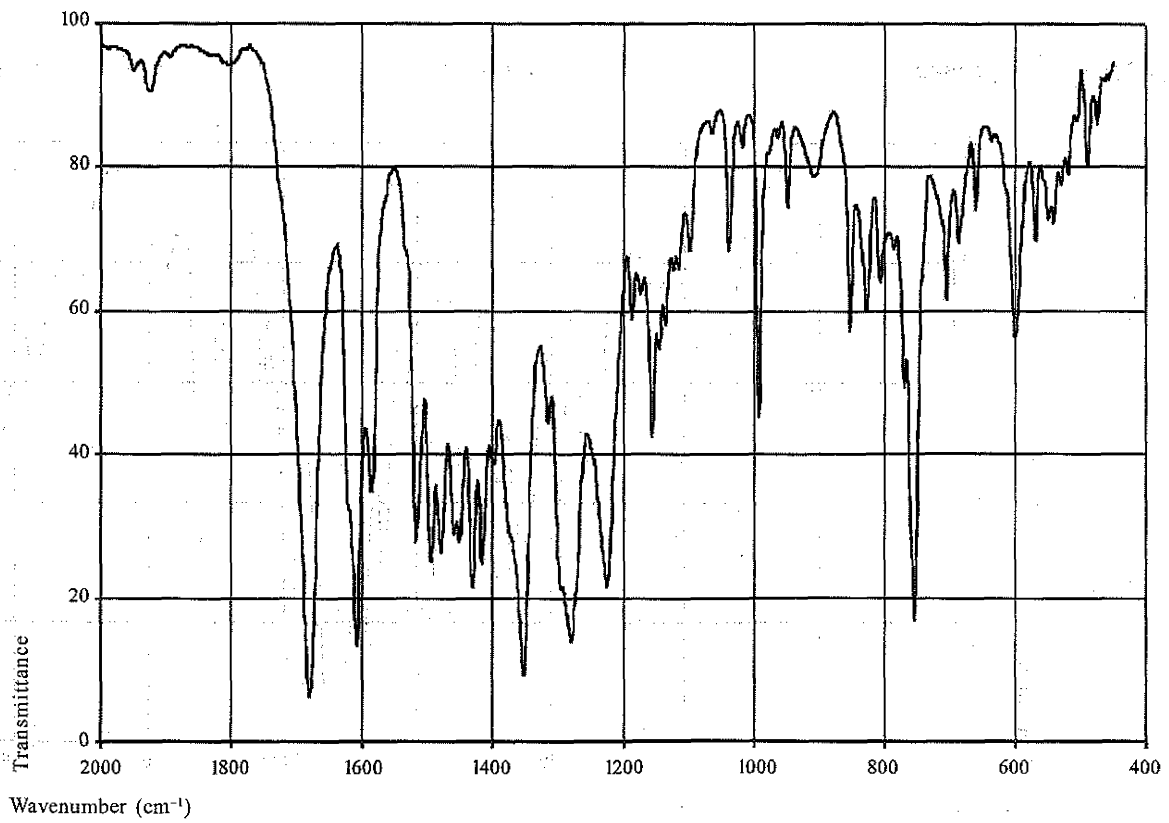
Daunorubicin Hydrochloride *Dispersive IR* Phase: KBr disc



Decoquinate *FTIR* Phase: KBr disc



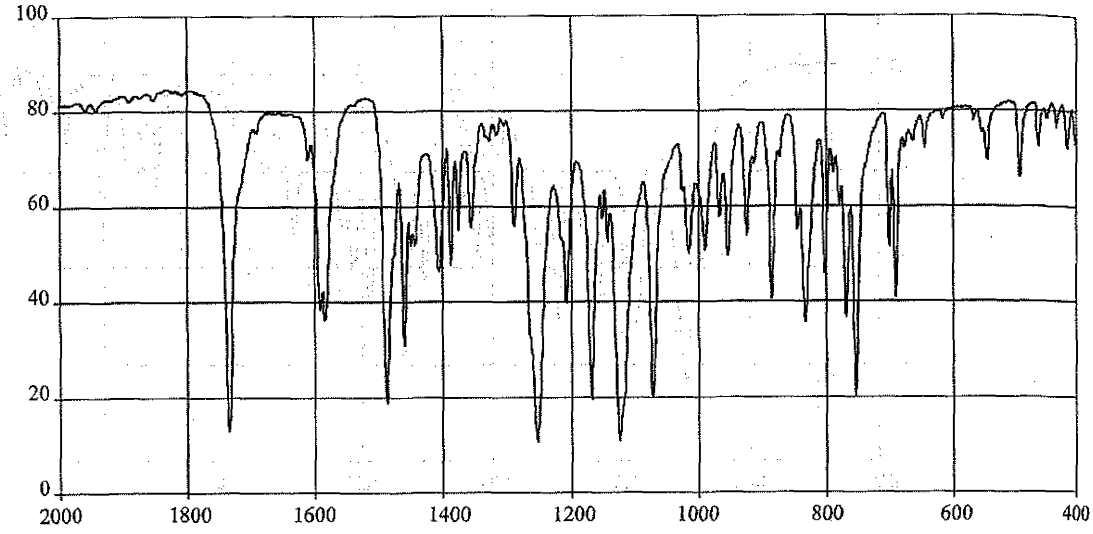
Deferasirox *FTIR* Phase: KBr disc



Deltamethrin

FTIR

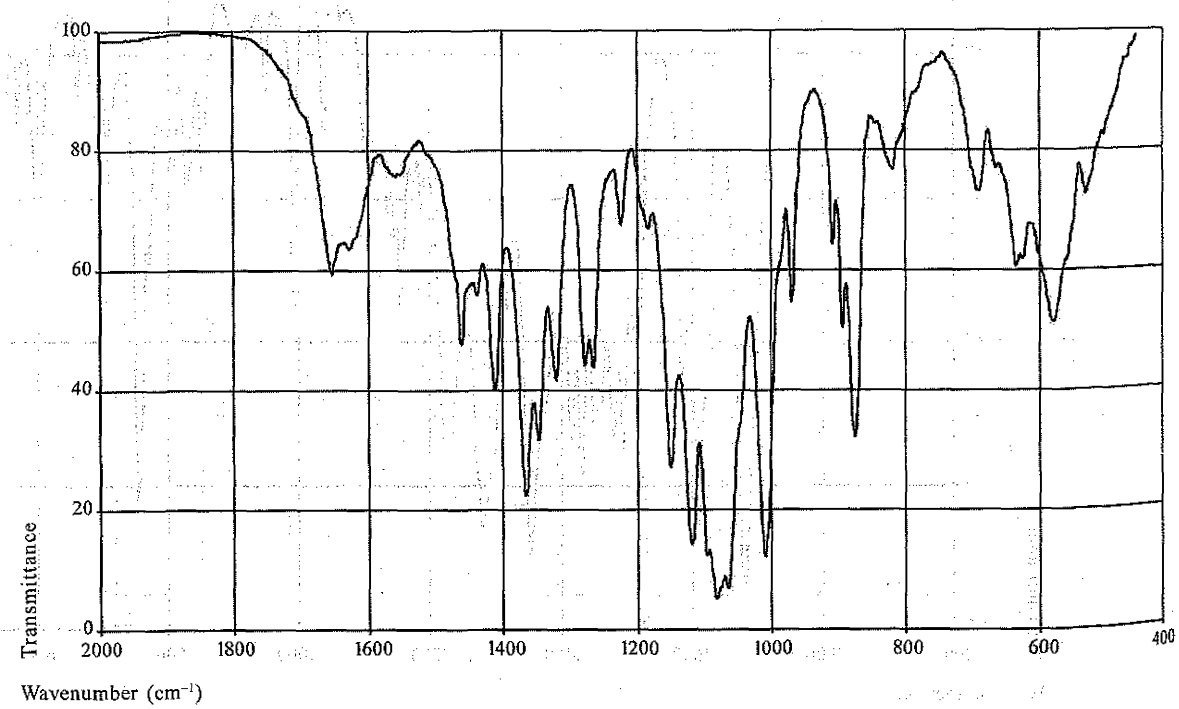
Phase: KBr disc



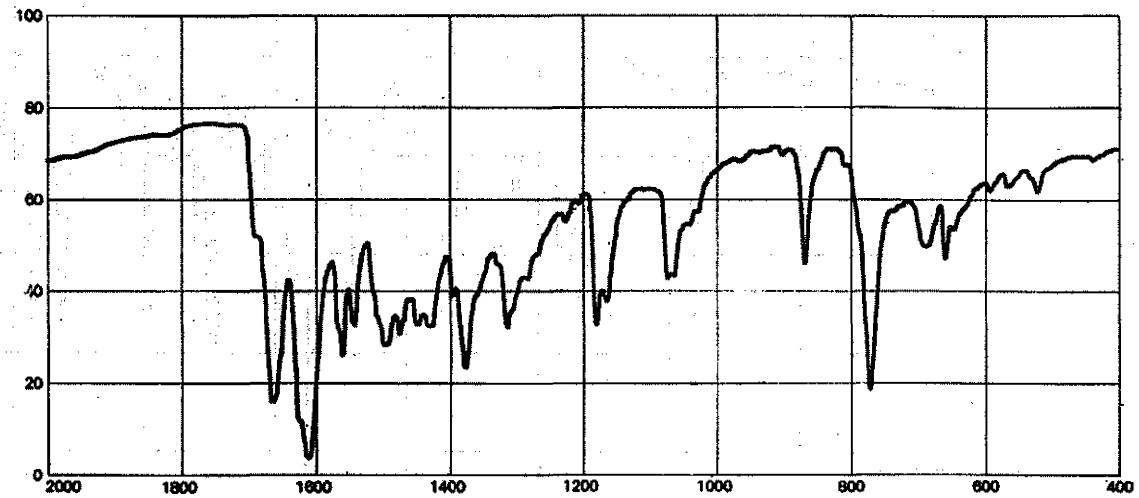
2-Deoxy-D-Glucose

FTIR

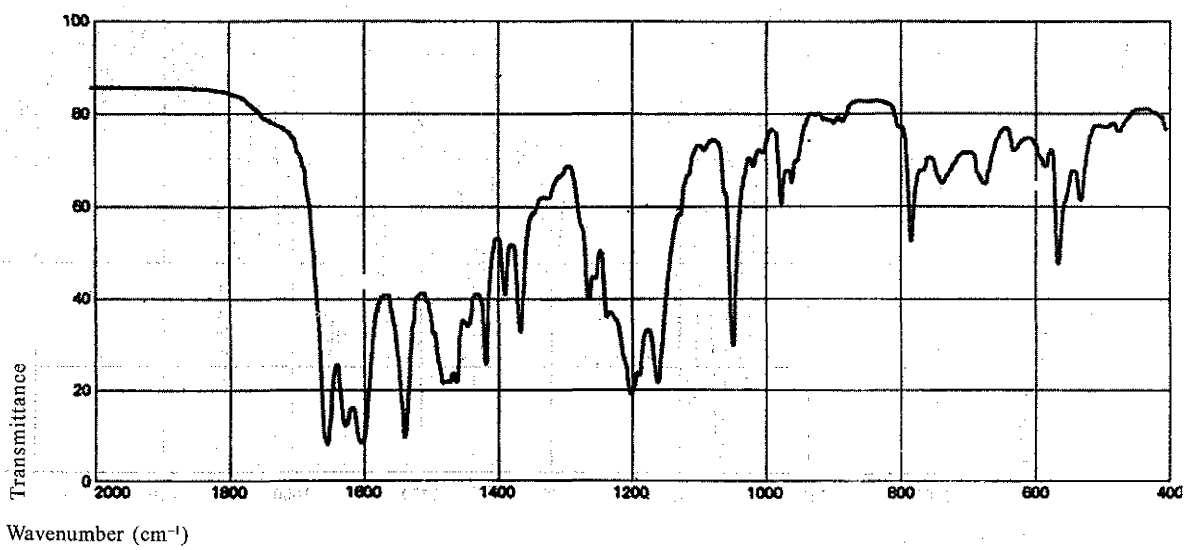
Phase: KBr disc



Dequalinium Chloride *Dispersive IR* Phase: KCl disc

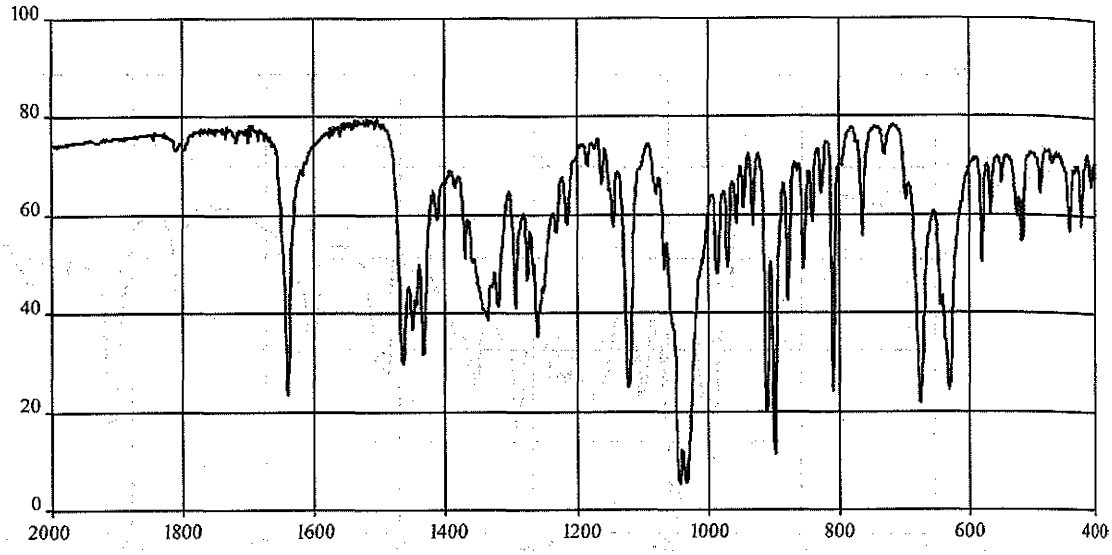


Desferrioxamine Mesylate *Dispersive IR* Phase: KBr disc



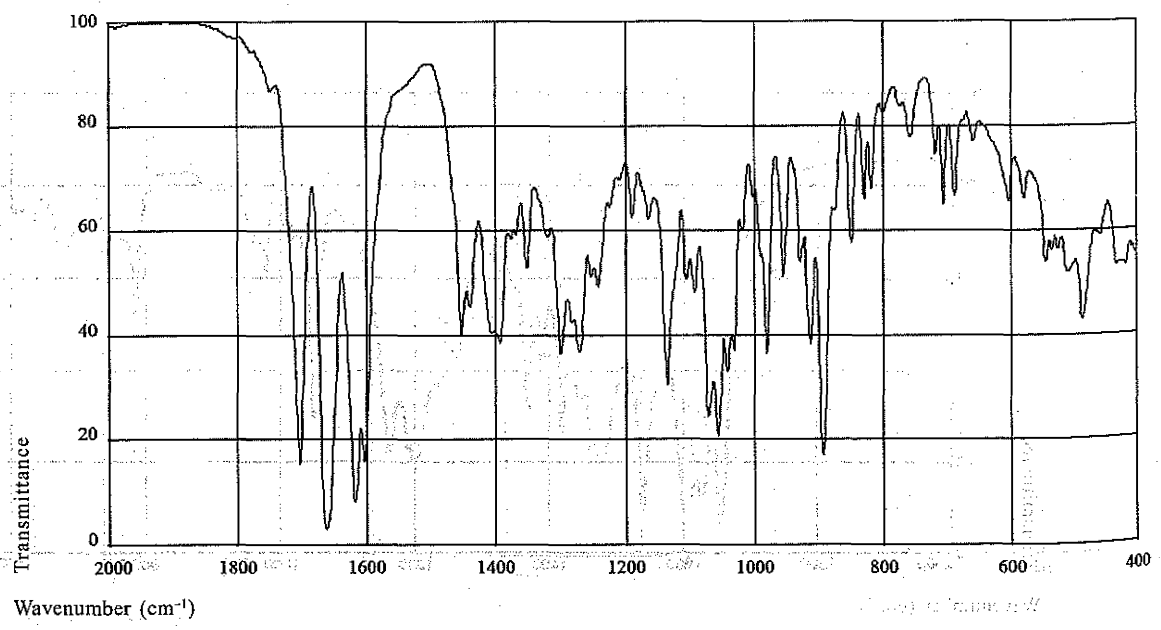
Desogestrel

FTIR Phase: KBr disc

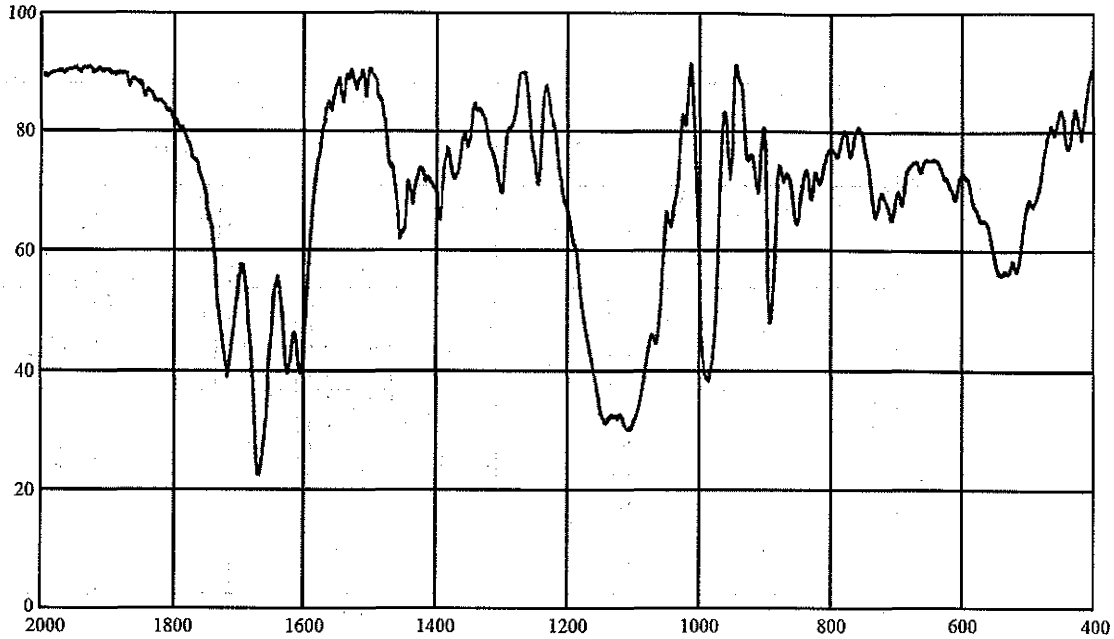


Dexamethasone

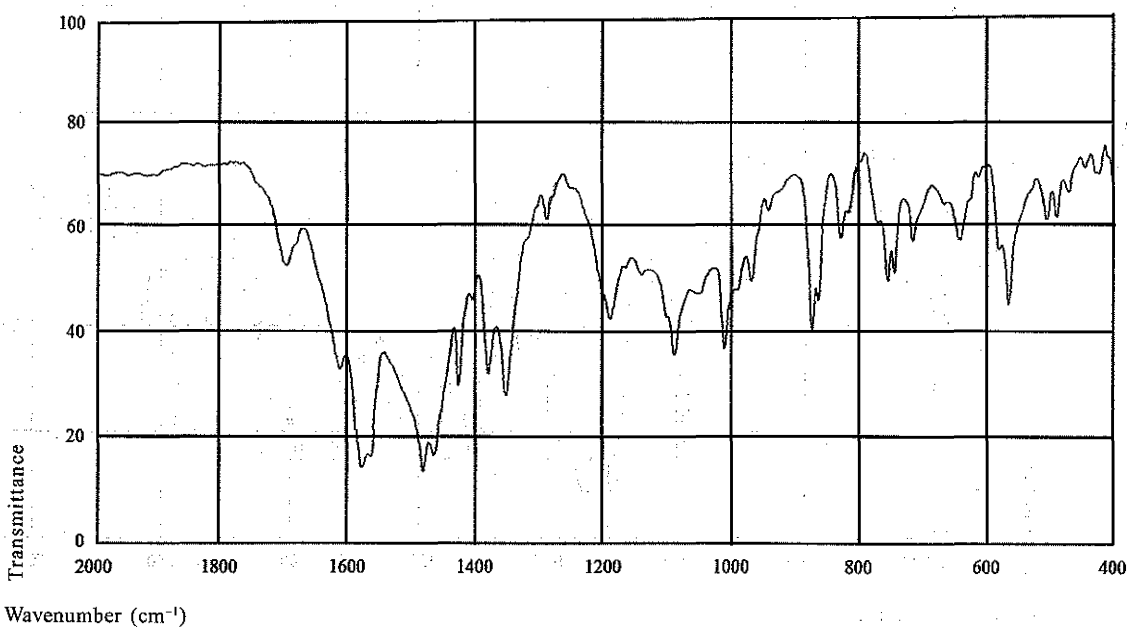
FTIR Phase: KBr disc



Dexamethasone Sodium Phosphate *FTIR* Phase: KBr disc



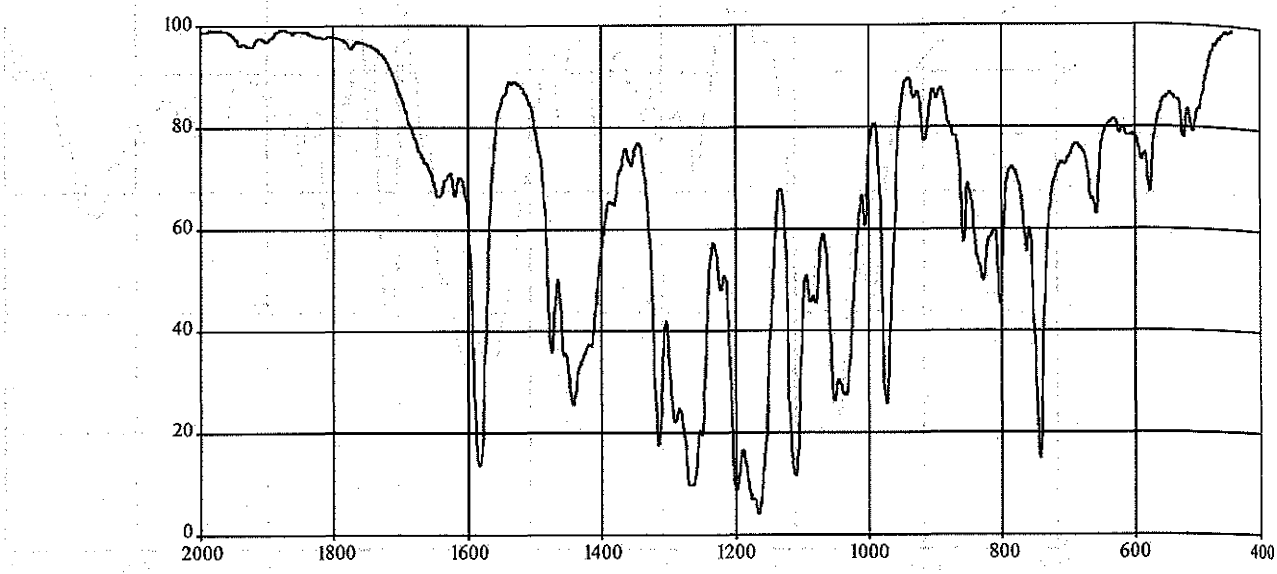
Dexchlorpheniramine Maleate *FTIR* Phase: KBr disc



Dexlansoprazole

FTIR

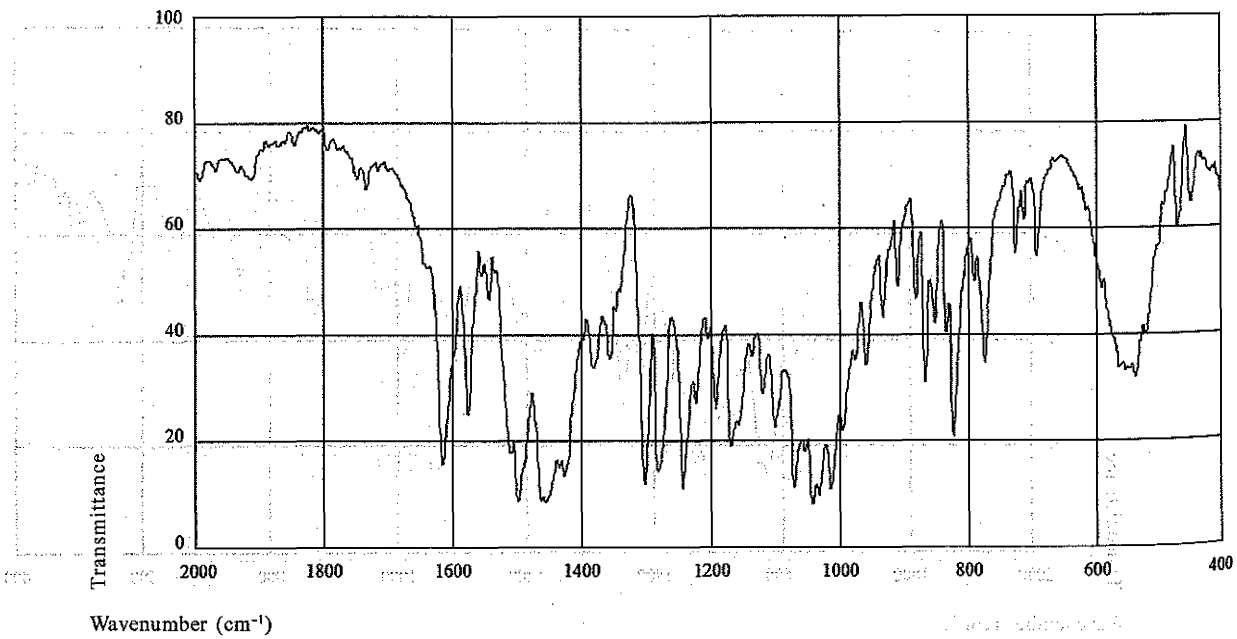
Phase: KBr disc



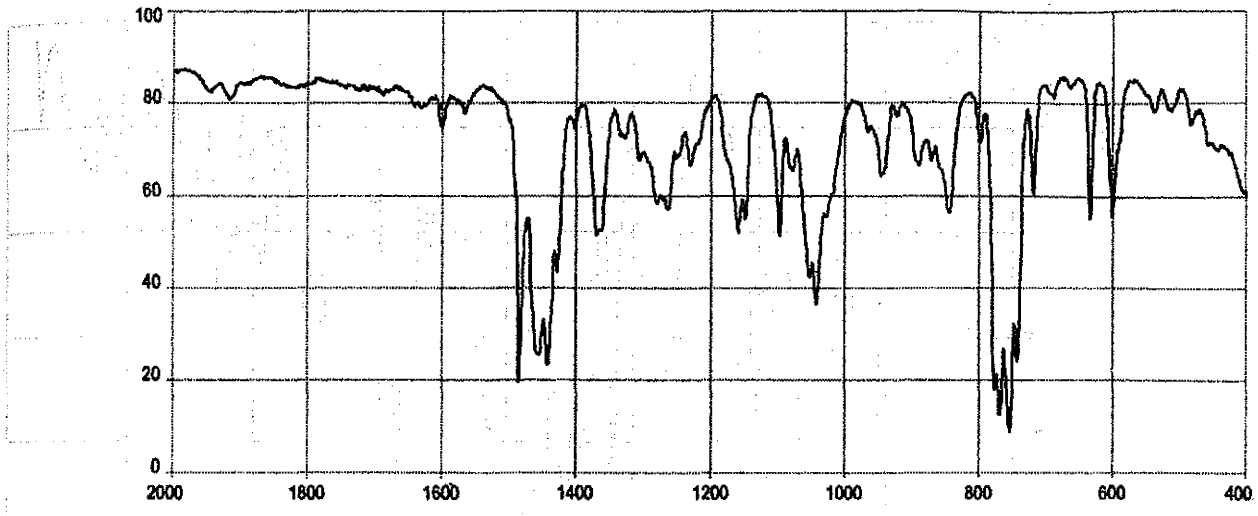
Dextromethorphan Hydrobromide

FTIR

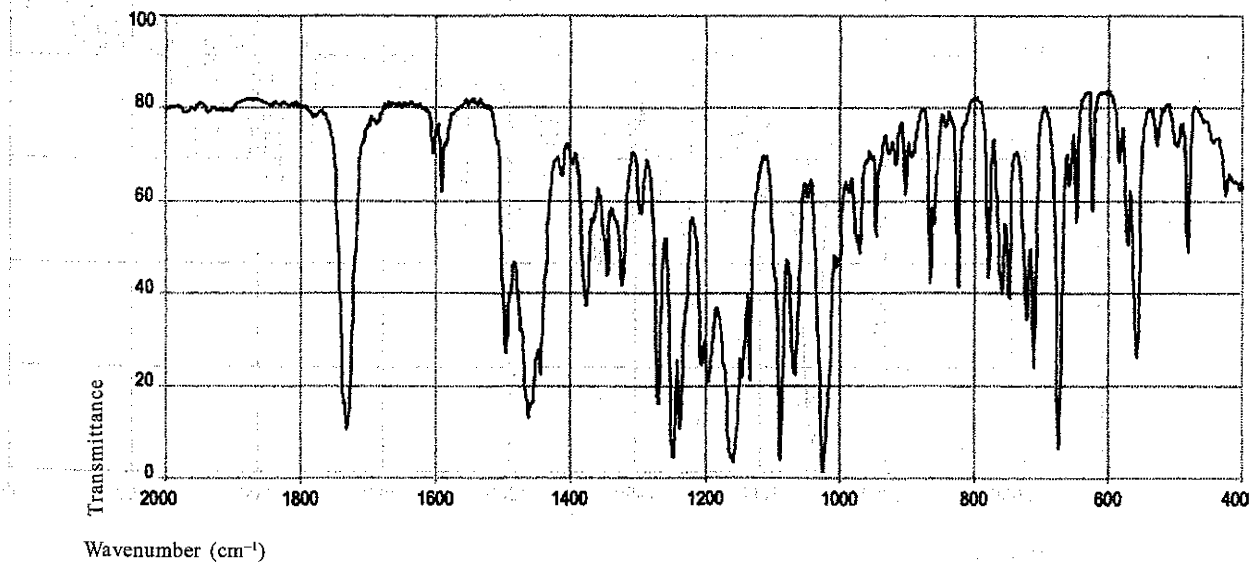
Phase: KBr disc



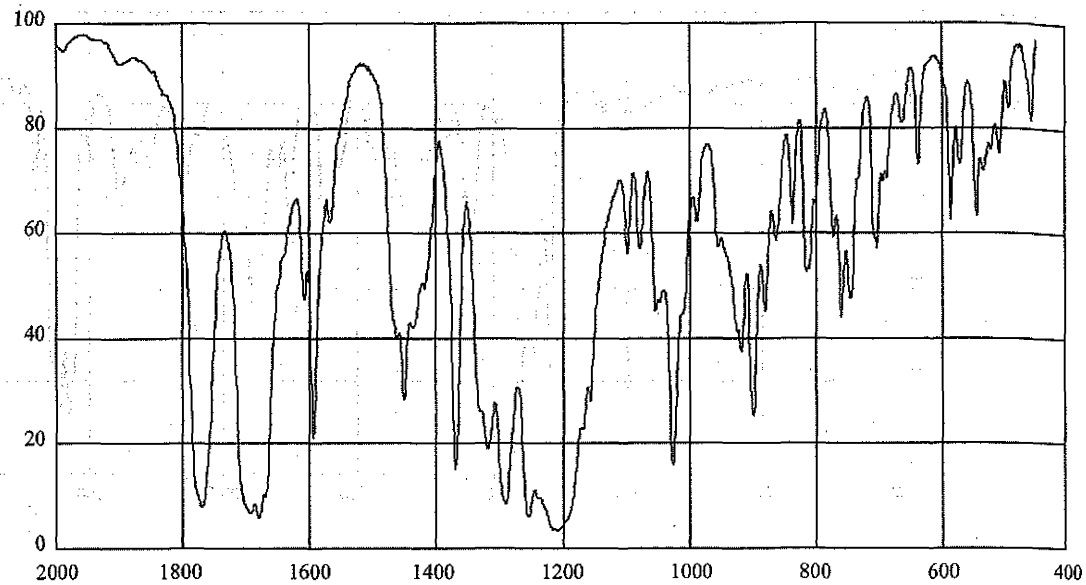
Dextropropoxyphene Hydrochloride *Dispersive IR* Phase: Thin film



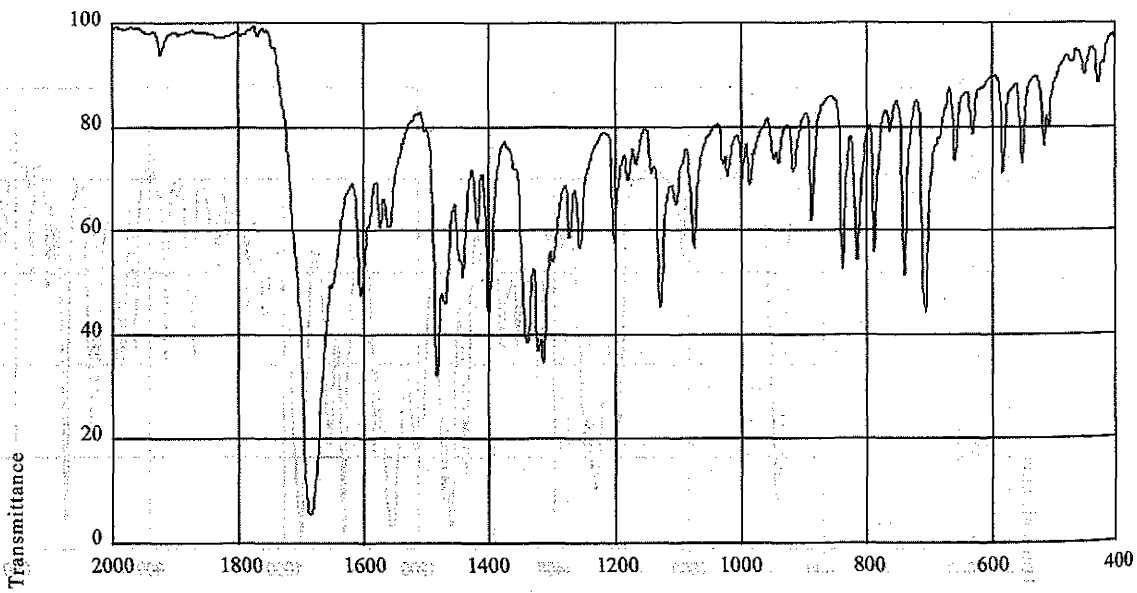
Dextropropoxyphene Napsilate *Dispersive IR* Phase: Liquid Paraffin mull



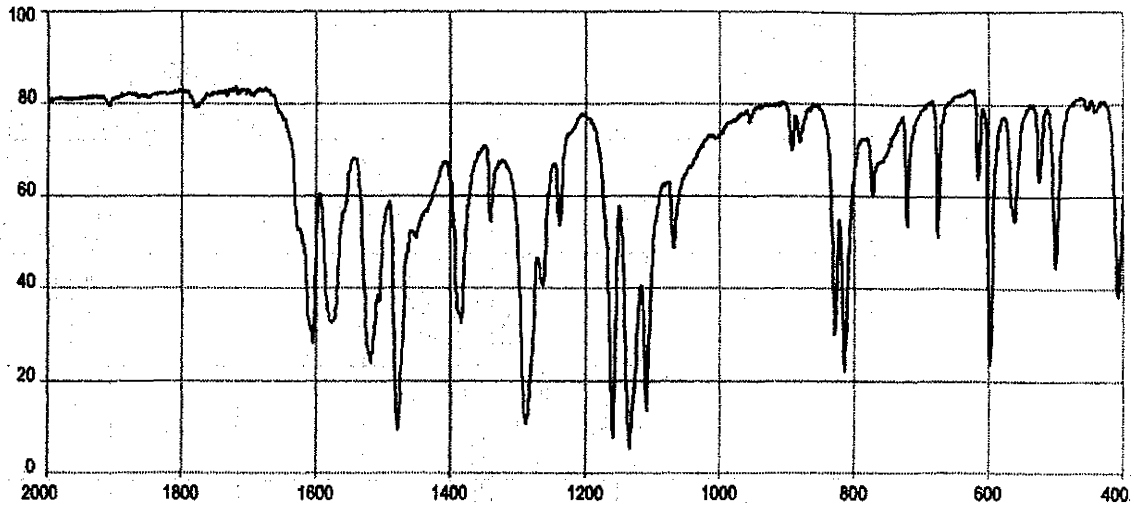
Diacerein *FTIR* Phase: KBr disc



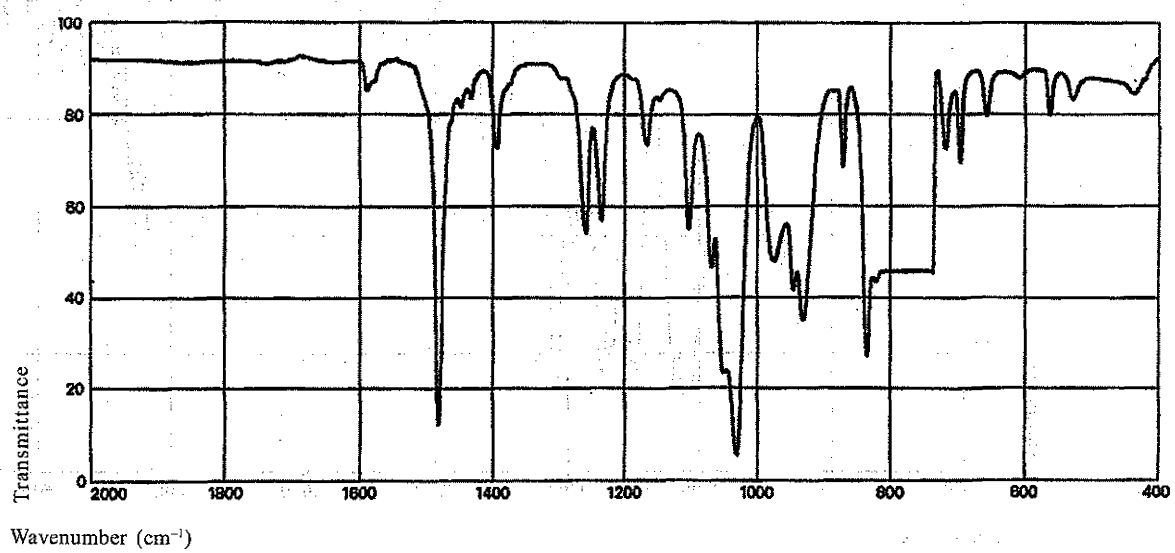
Diazepam *FTIR* Phase: KBr disc



Diazoxide *Dispersive IR* Phase: KBr disc



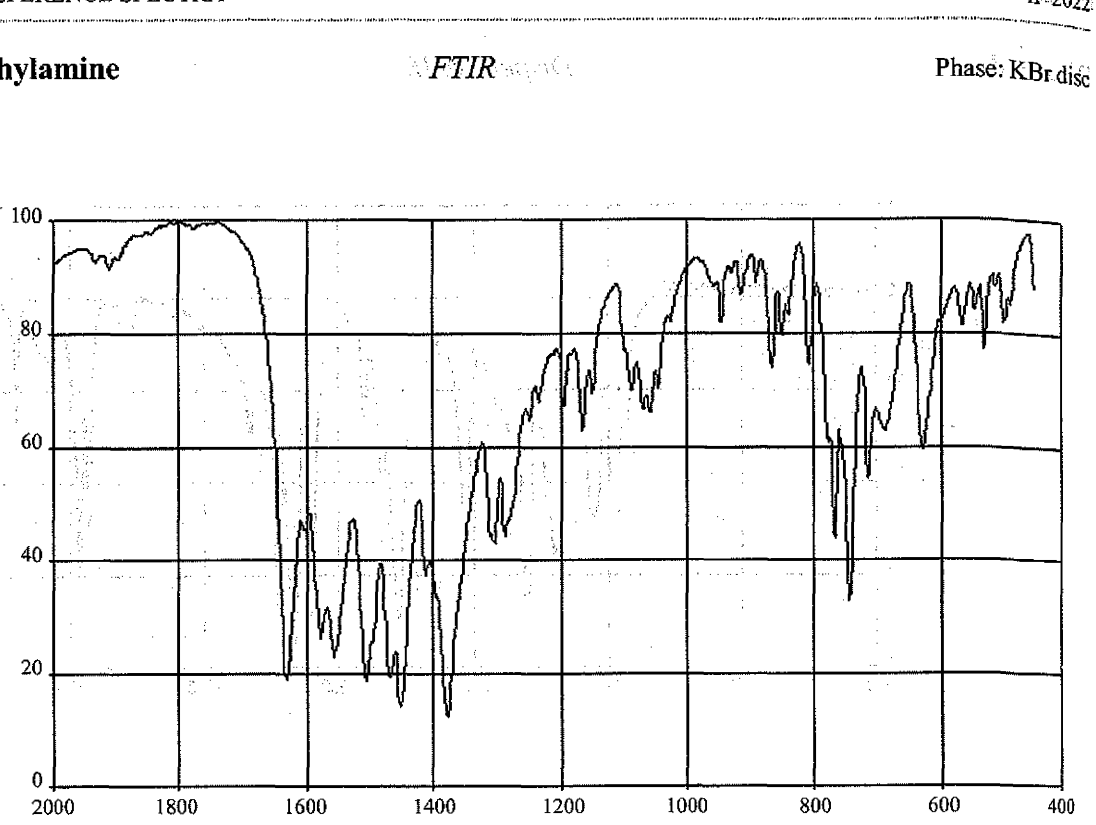
Dichlofenthion *Dispersive IR* Phase: 4 per cent w/v solution in CCl_4 Thickness 0.1 mm



Diclofenac Diethylamine

FTIR

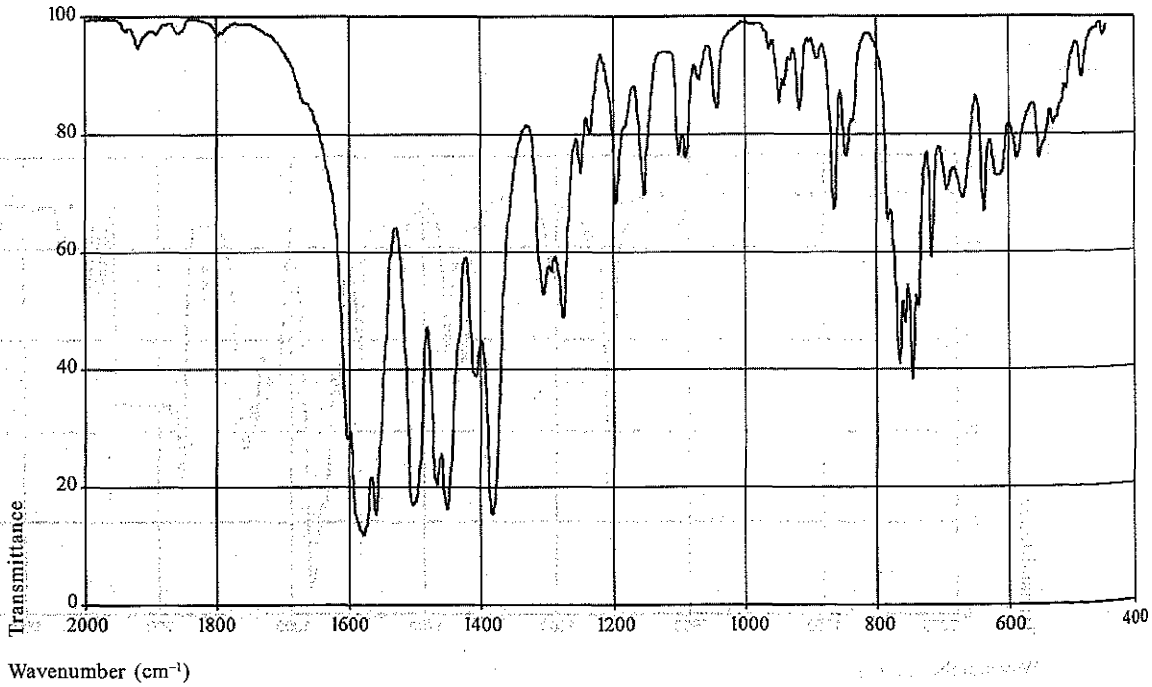
Phase: KBr disc



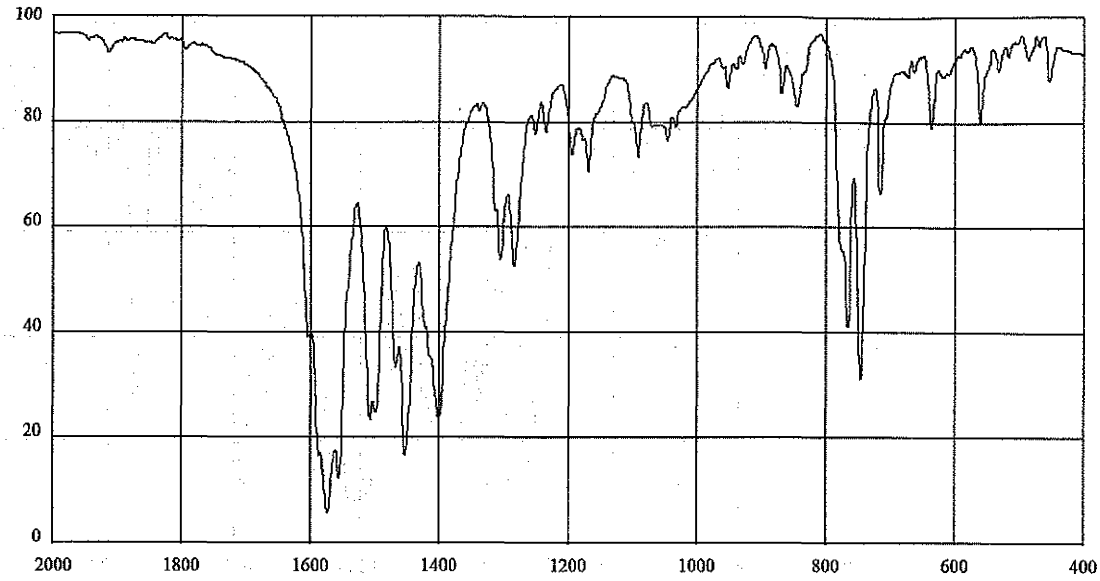
Diclofenac Potassium

FTIR

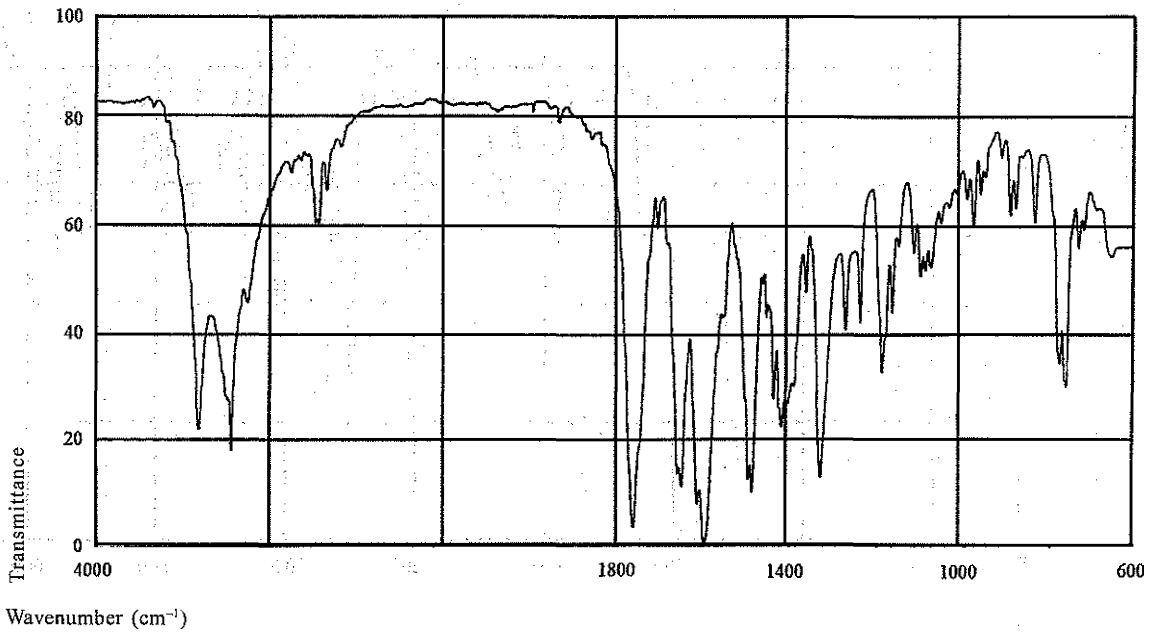
Phase: KBr disc



Diclofenac Sodium *FTIR* Phase: KBr disc



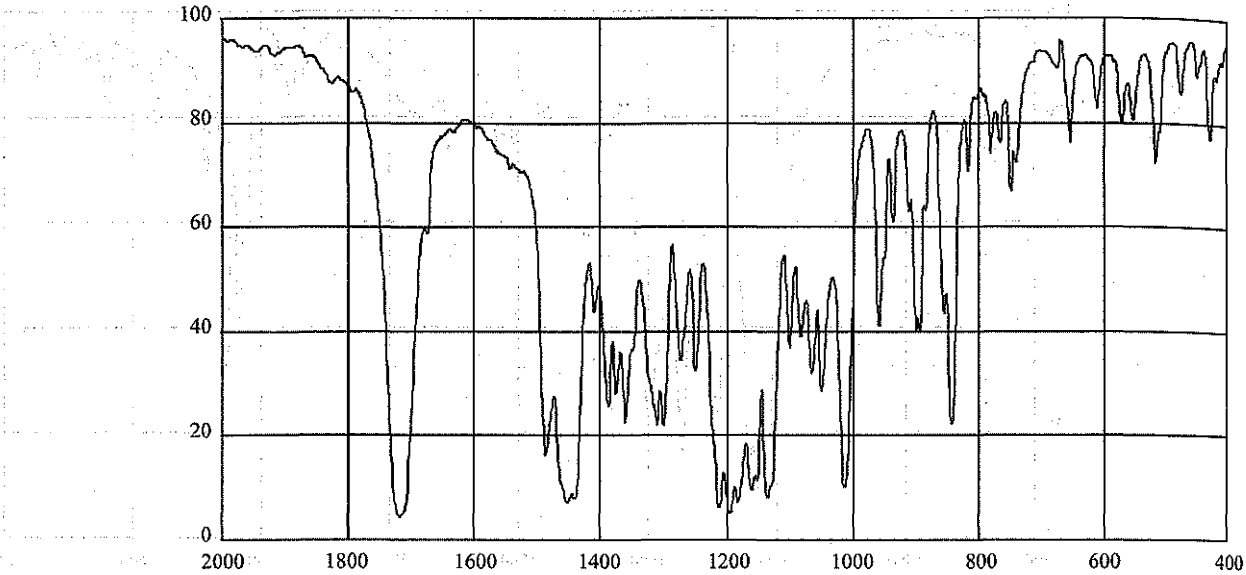
Dicloxacillin Sodium *Dispersive IR* Phase: KCl disc



Dicyclomine Hydrochloride

FTIR

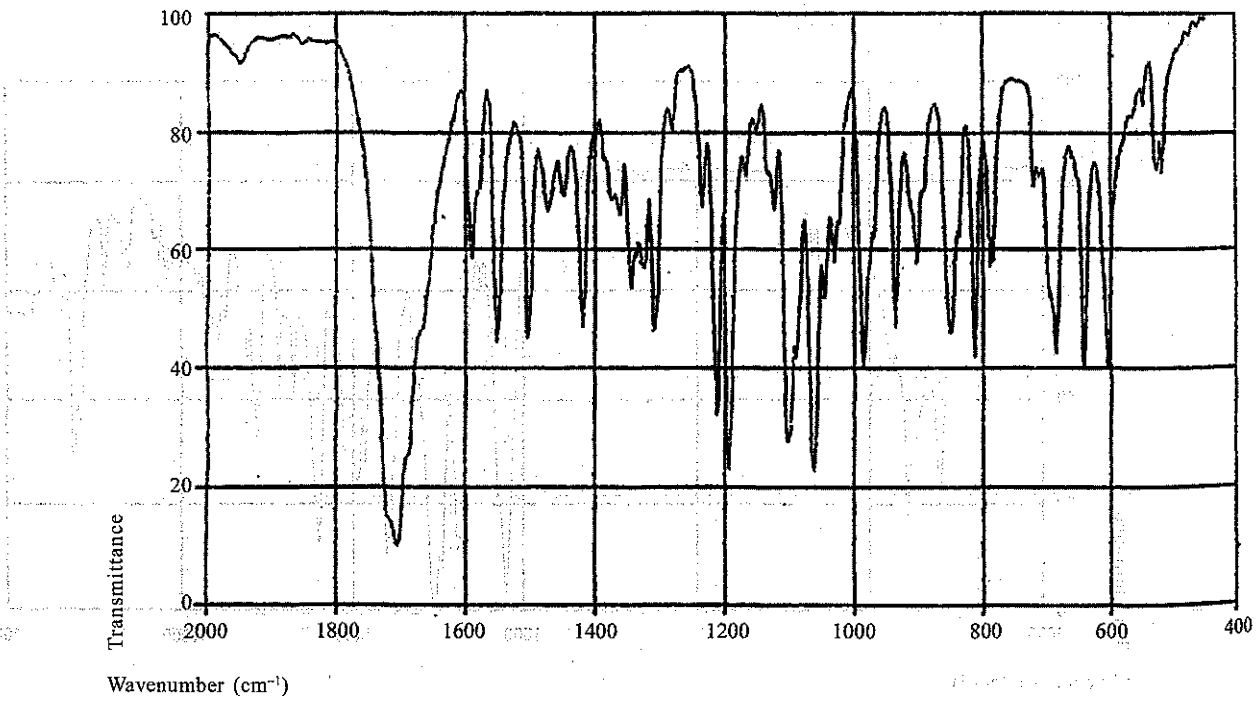
Phase: KCl disc



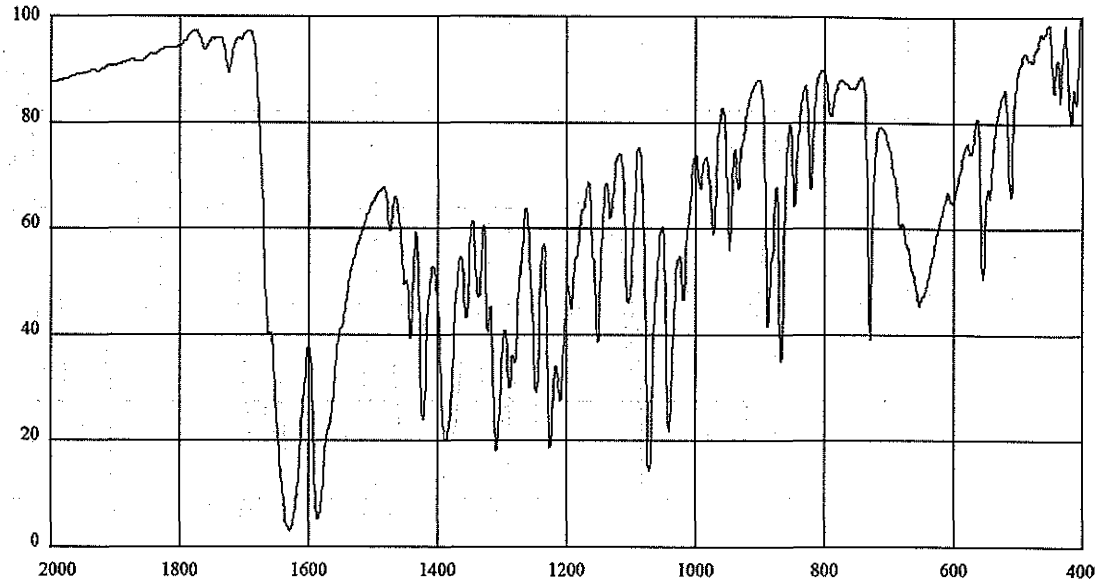
Didanosine

FTIR

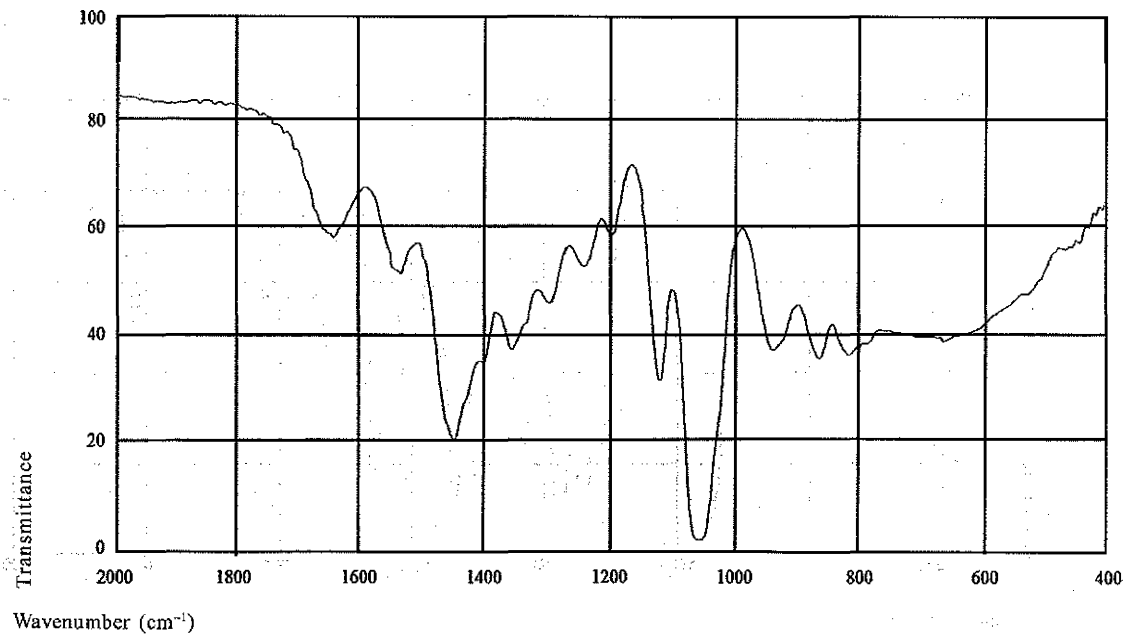
Phase: KBr disc



Dienogest *FTIR* Phase: KBr disc



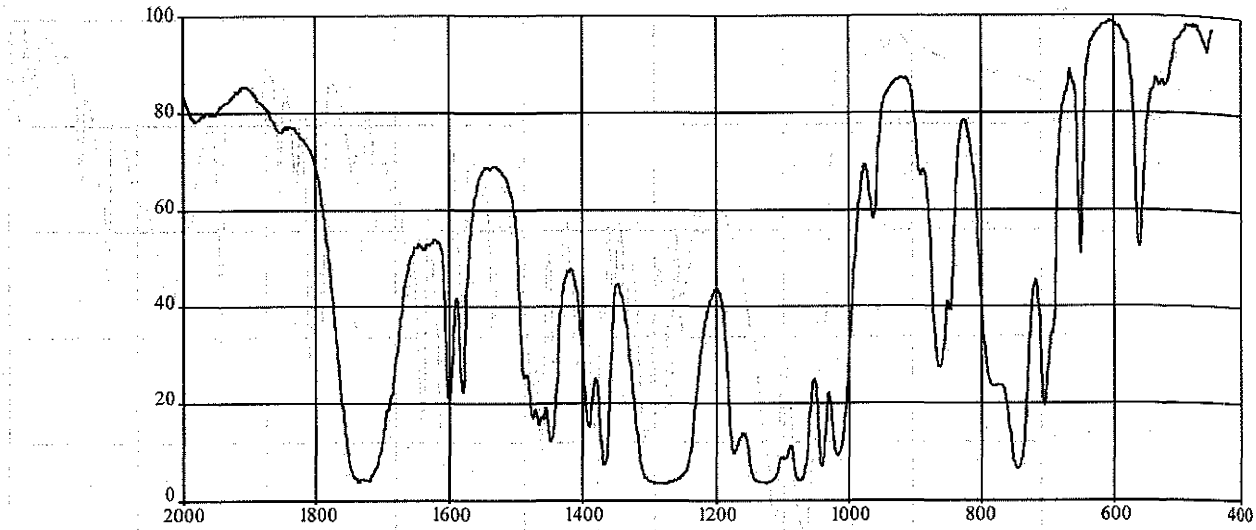
Diethanolamine *FTIR* Phase: KBr disc



Diethyl Phthalate

FTIR

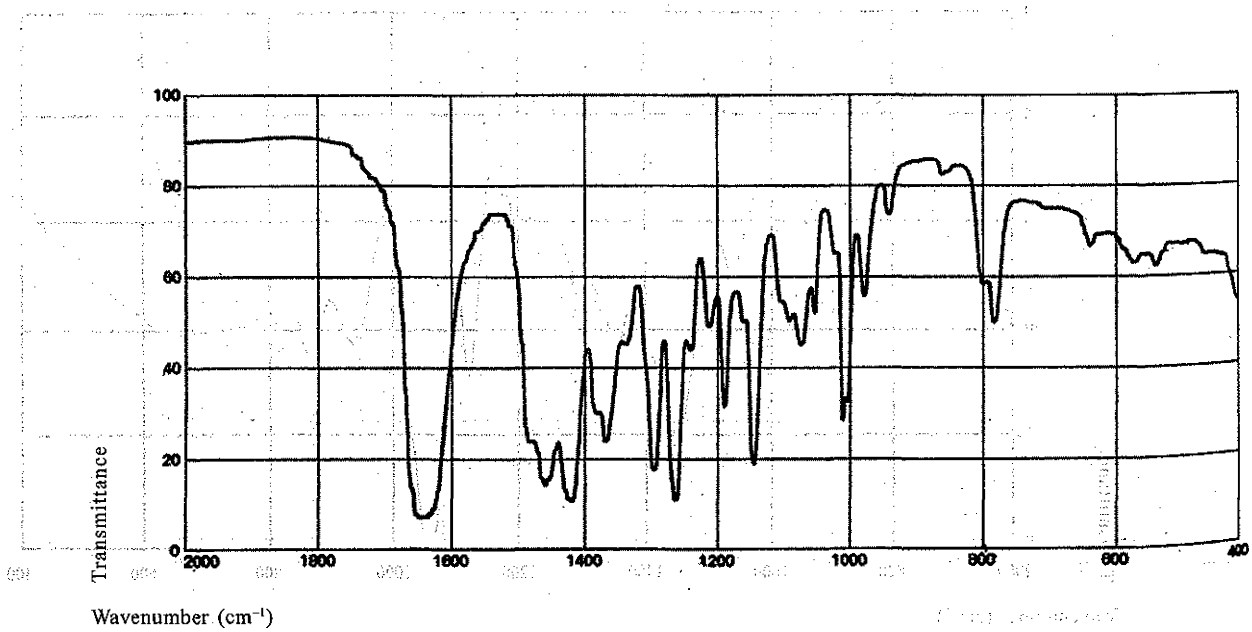
Phase: KBr disc



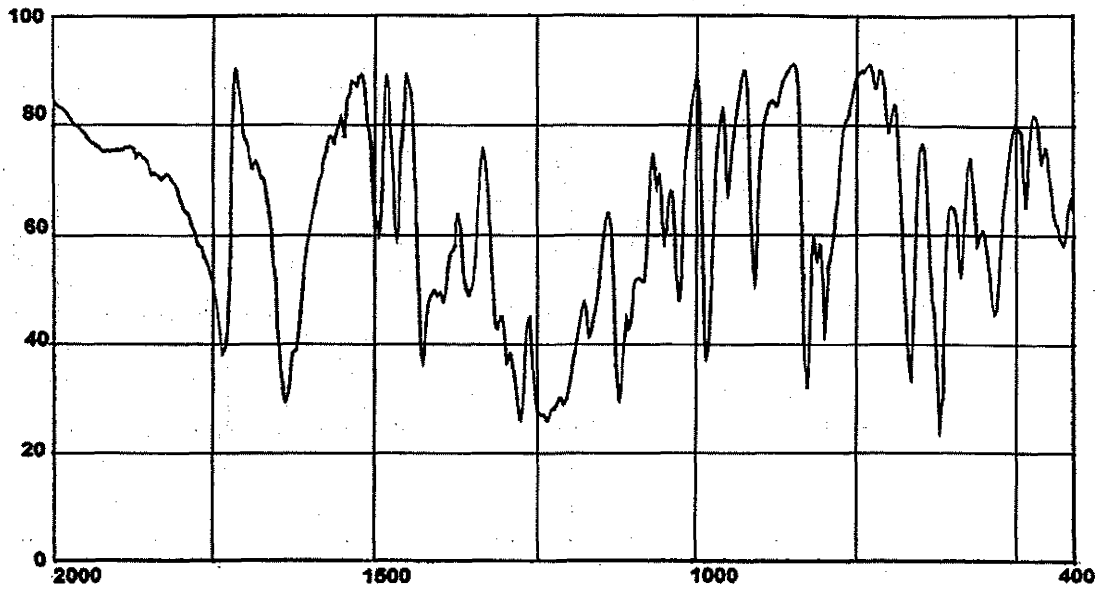
Diethylcarbamazine

Dispersive IR

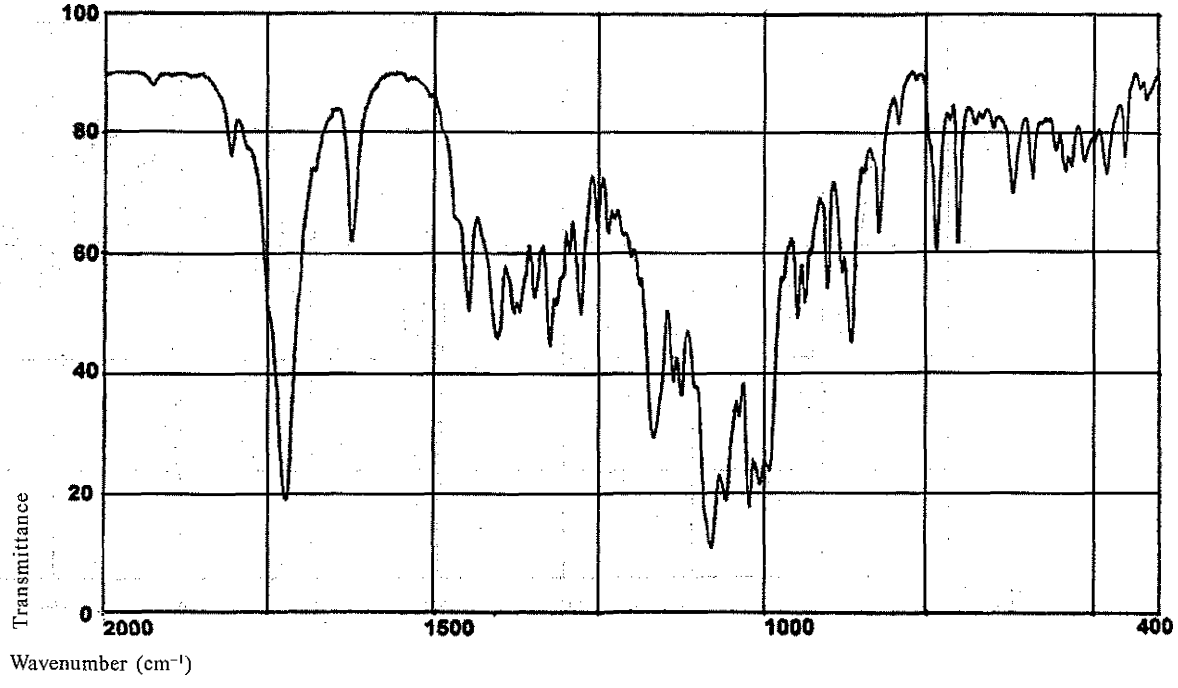
Phase: Thin film



Diethylcarbamazine Citrate *FTIR* Phase: KBr disc



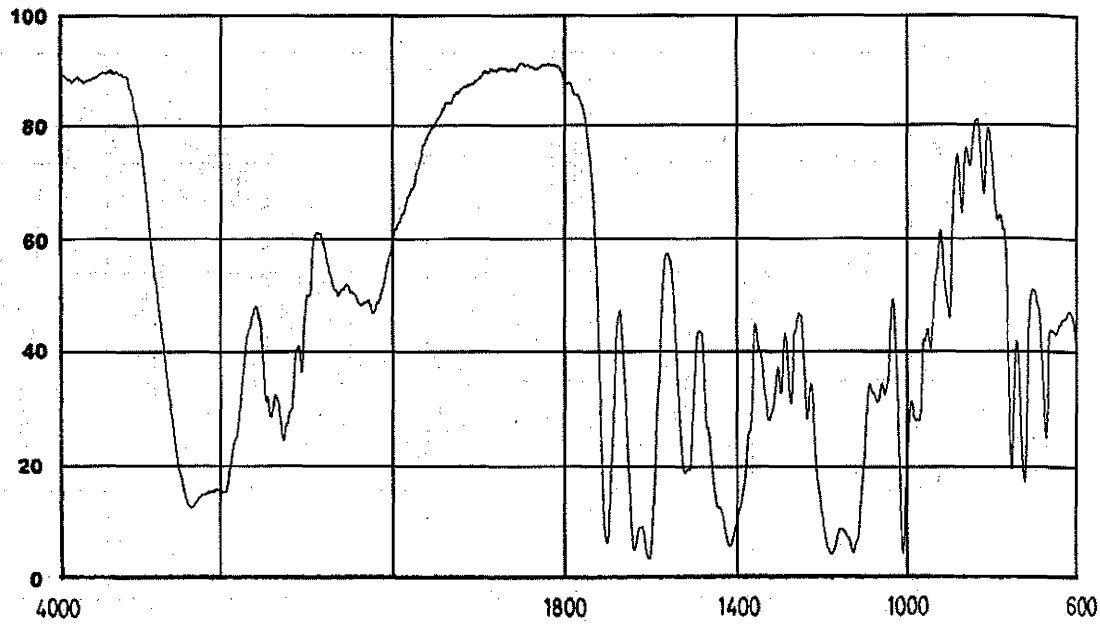
Digoxin *FTIR* Phase: KBr disc



Dihydroergocristine Mesylate

FTIR

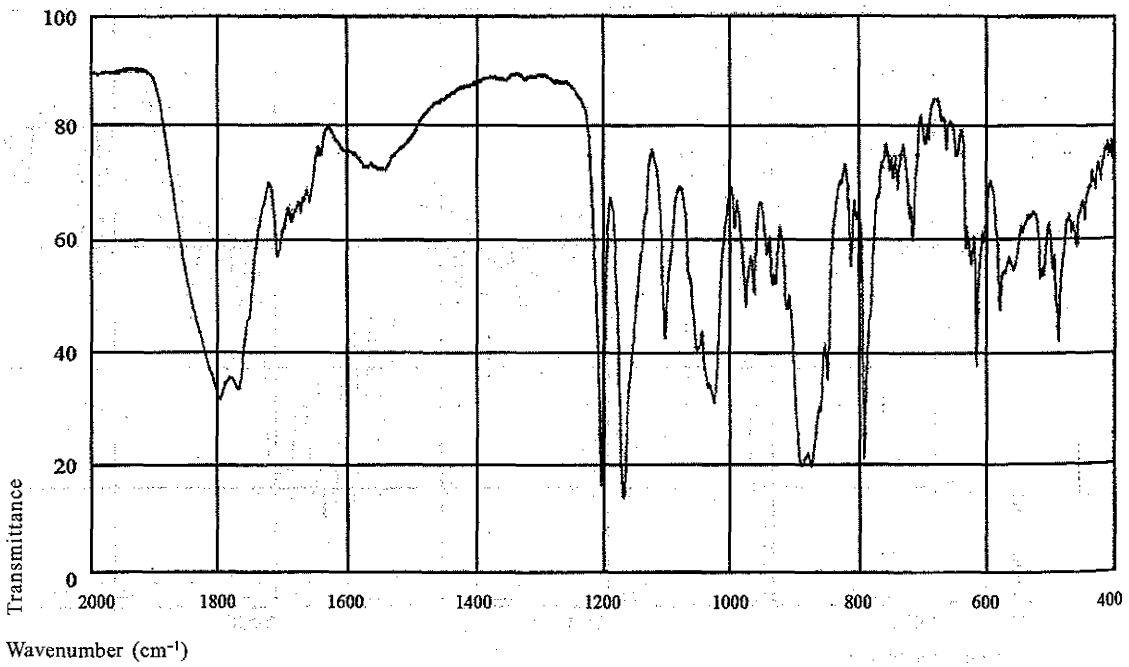
Phase: KBr disc



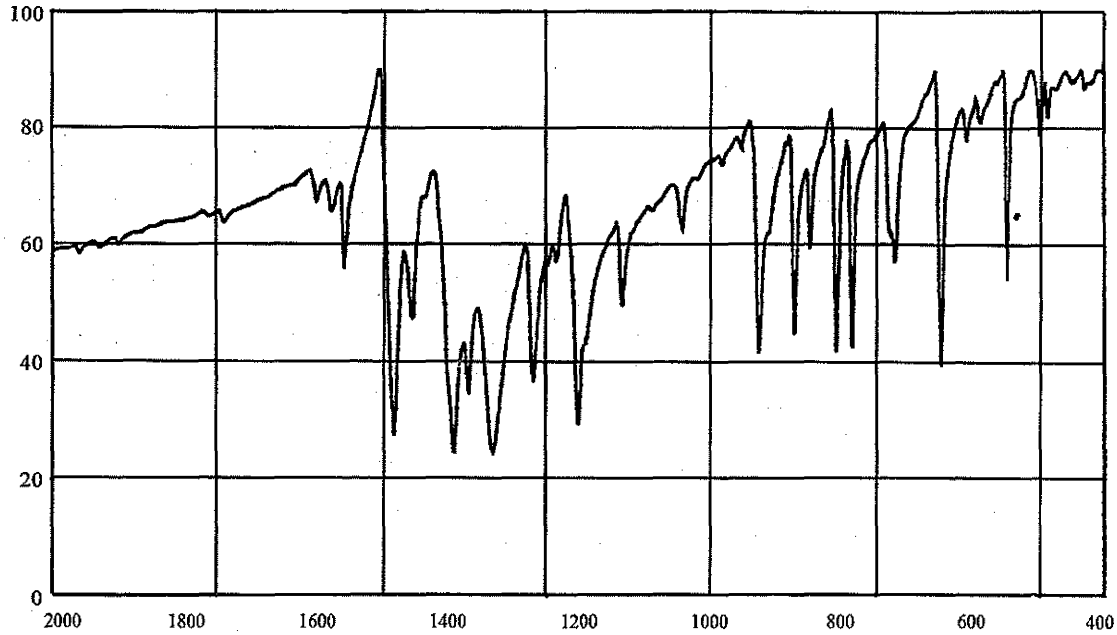
Dihydroergotamine Mesylate

FTIR

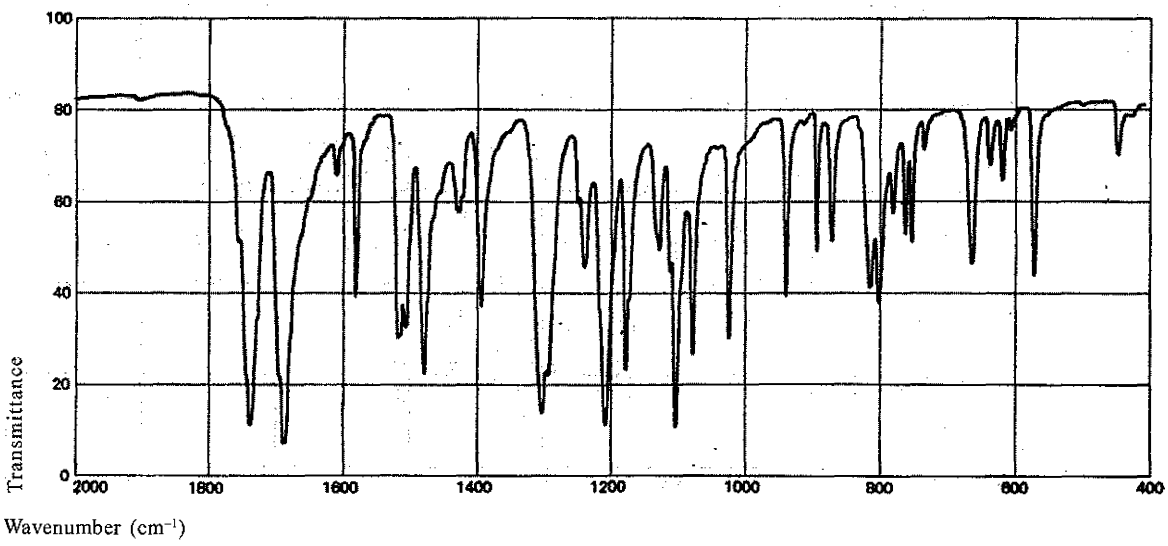
Phase: KBr disc



Diiodohydroxyquinoline *FTIR* Phase: KBr disc



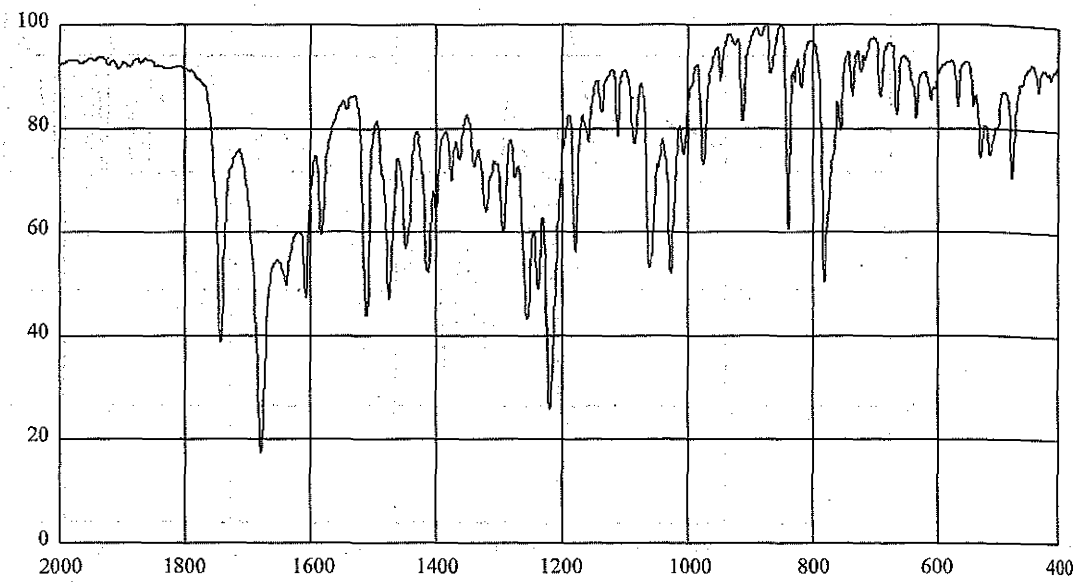
Diloxanide Furoate *Dispersive IR* Phase: KBr disc



Diltiazem Hydrochloride

FTIR

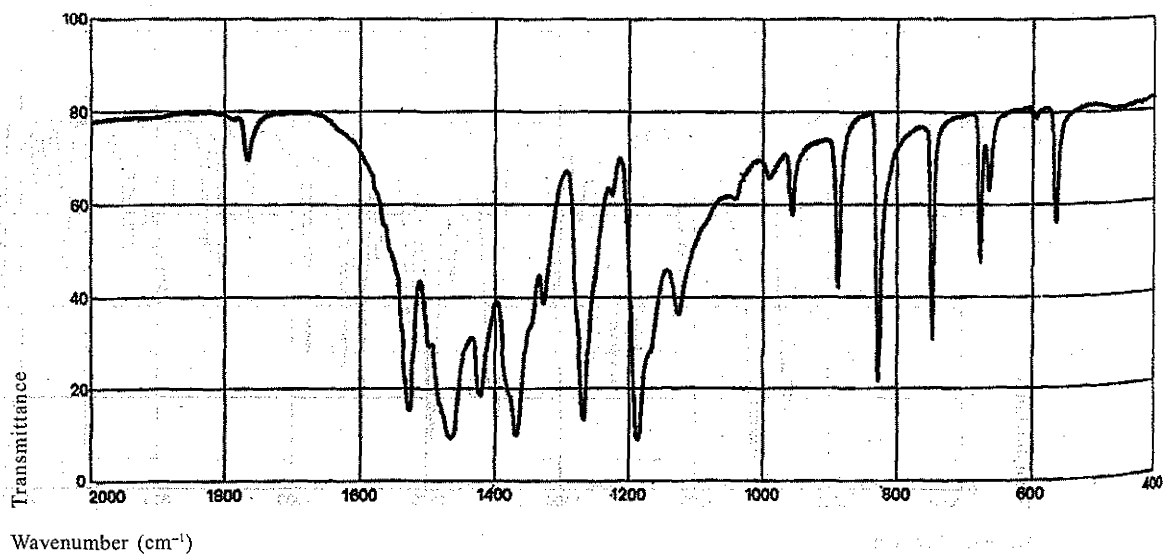
Phase: KBr disc



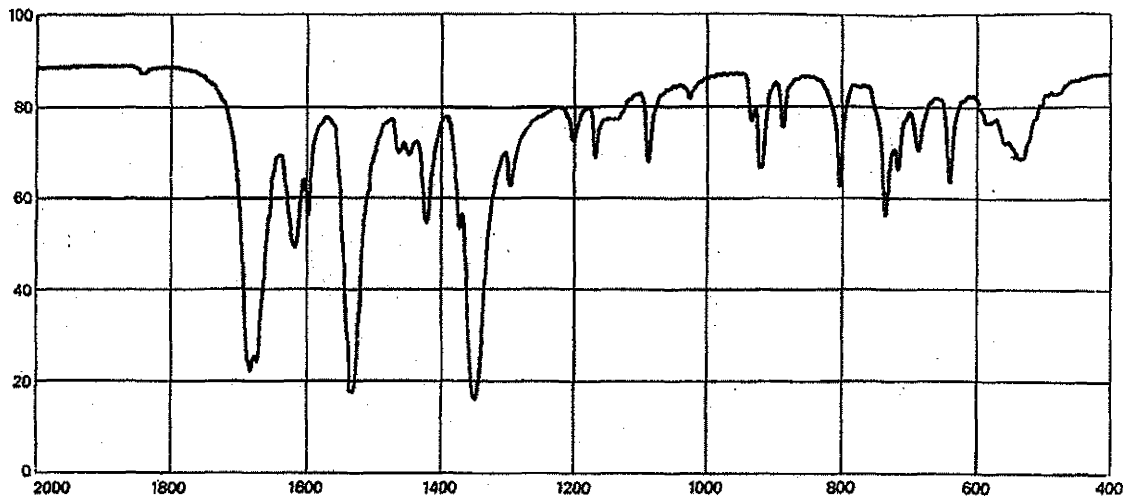
Dimetridazole

Dispersive IR

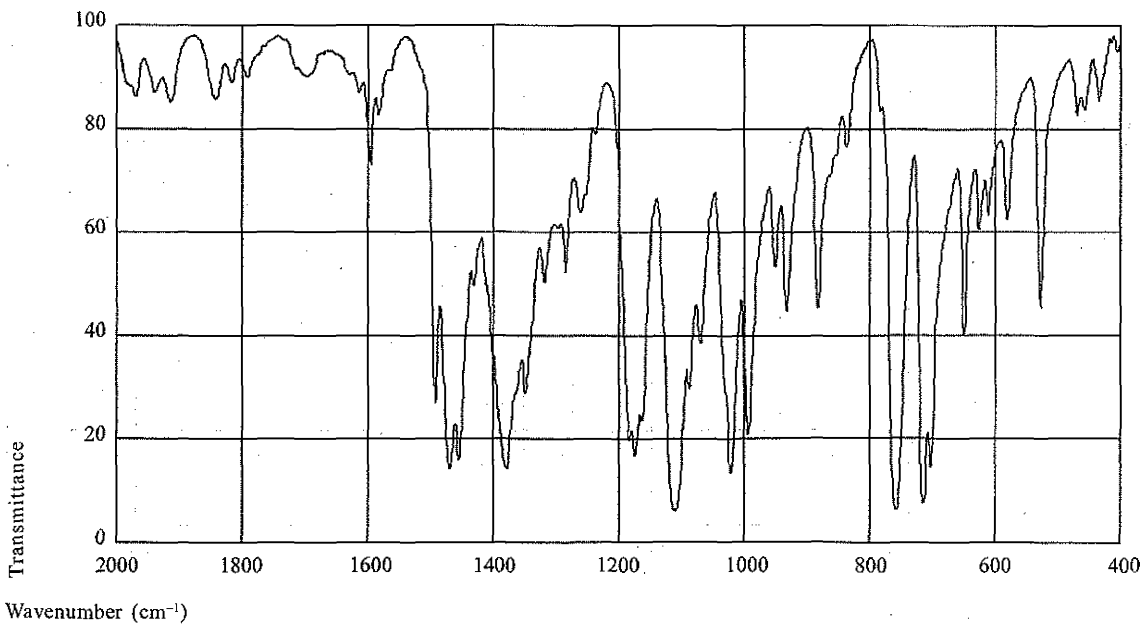
Phase: KBr disc



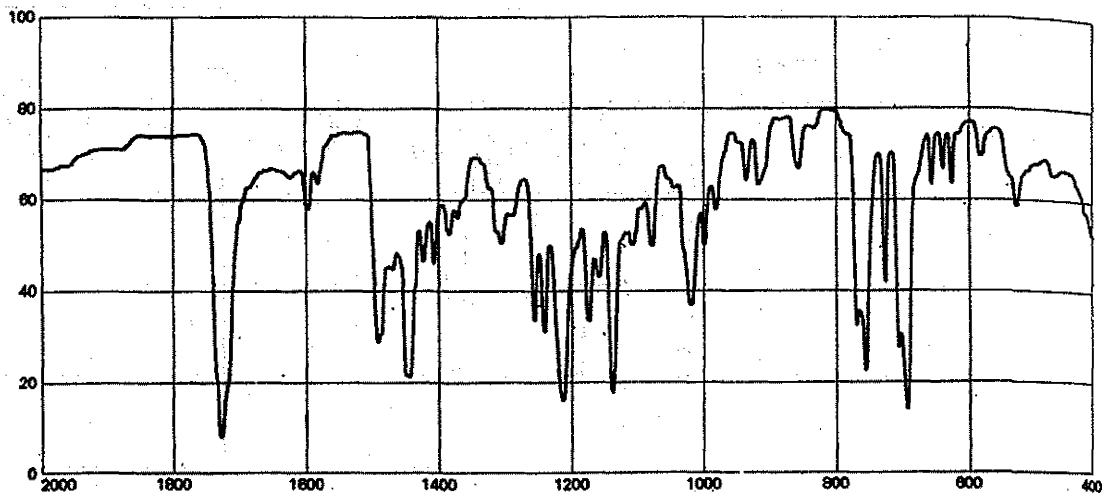
Dinitolmide *Dispersive IR* Phase: KBr disc



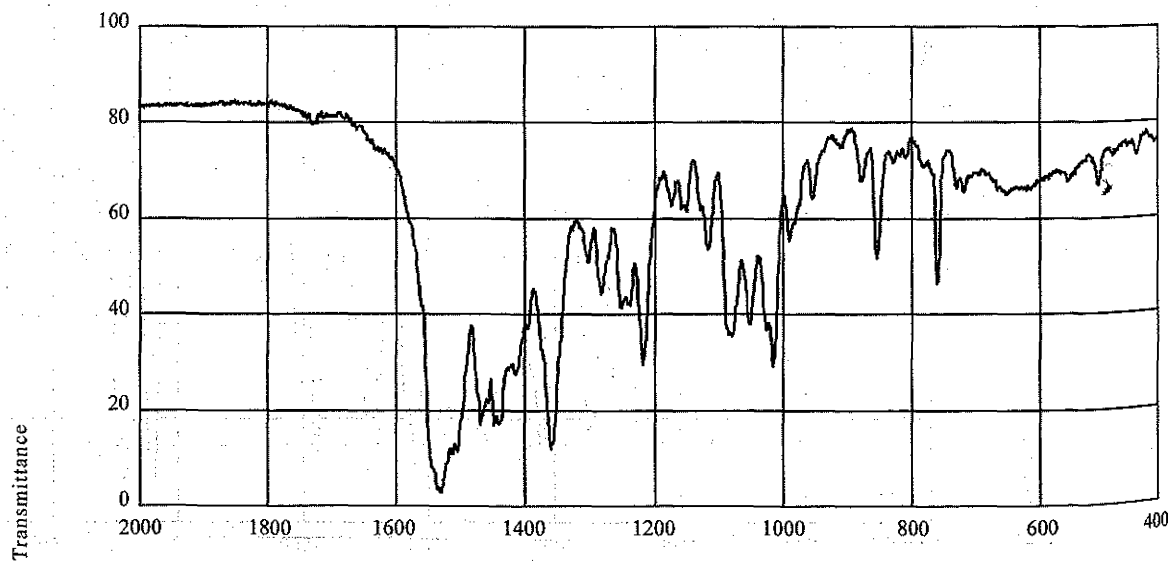
Diphenhydramine Hydrochloride *FTIR* Phase: KBr disc



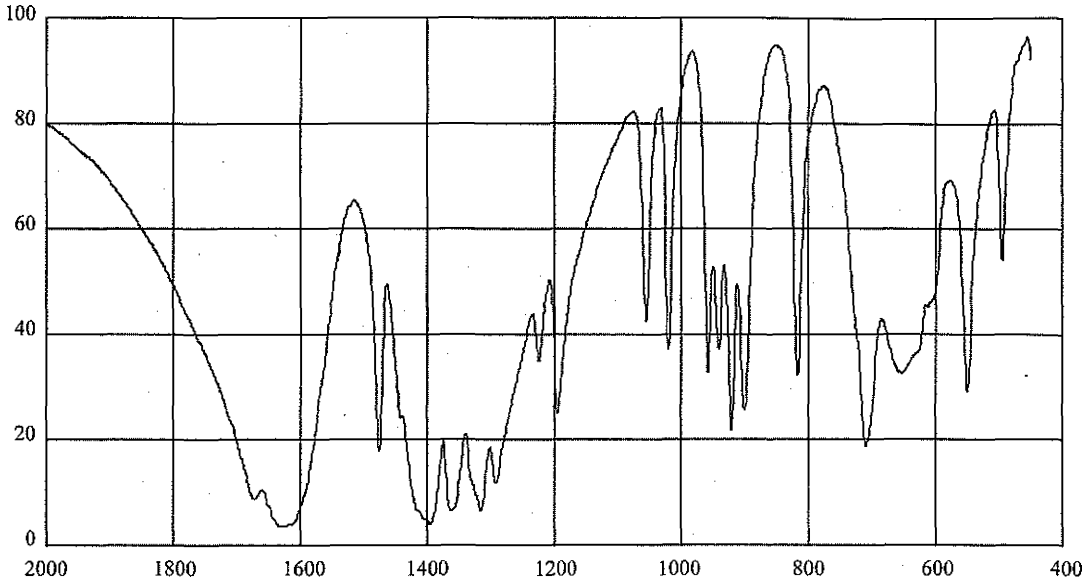
Diphenoxylate Hydrochloride *Dispersive IR* Phase: KBr disc



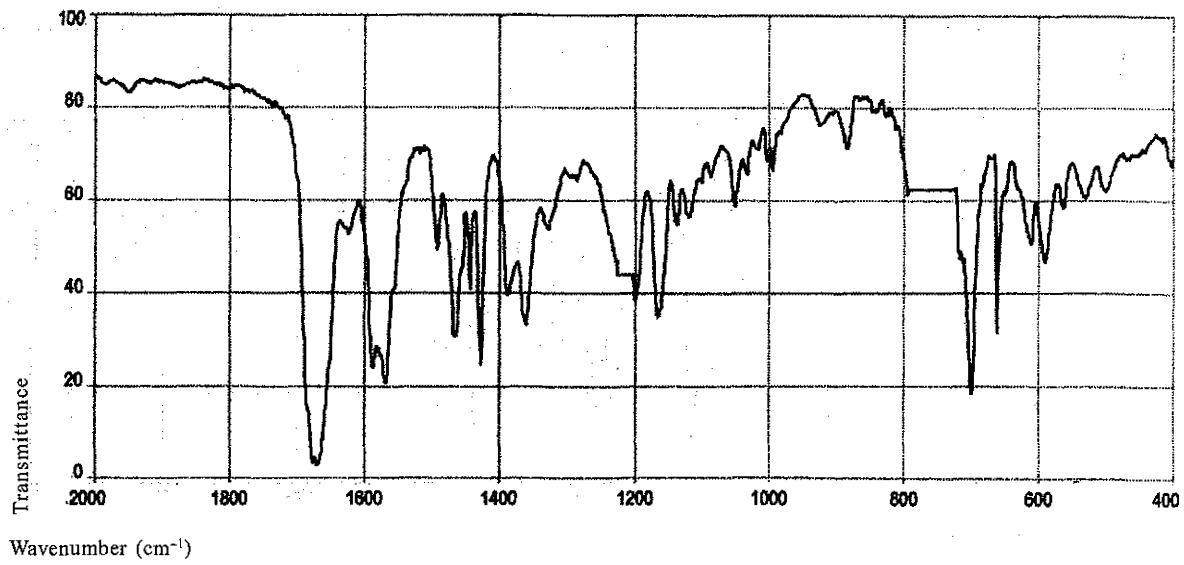
Dipyridamole *FTIR* Phase: KBr disc



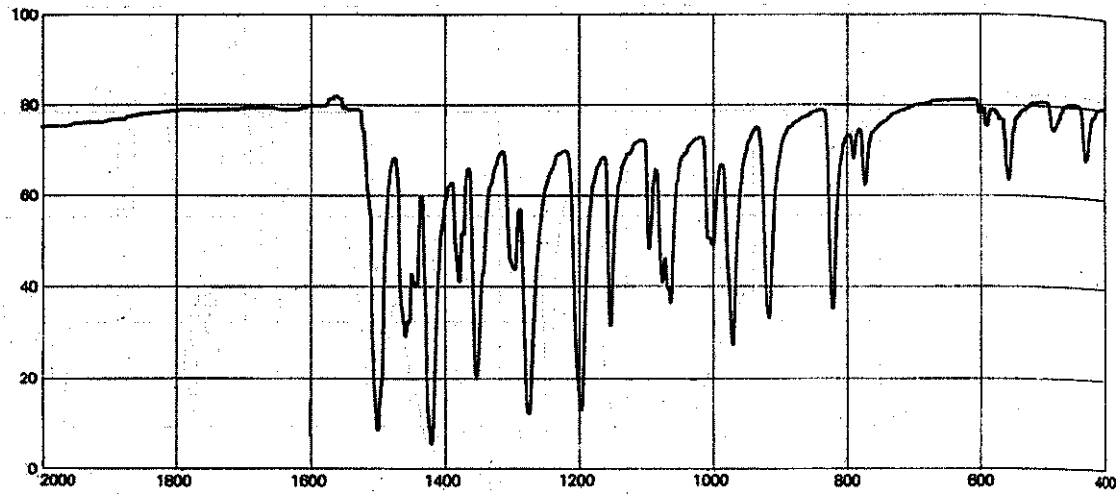
Disodium Edetate *FTIR* Phase: KBr disc



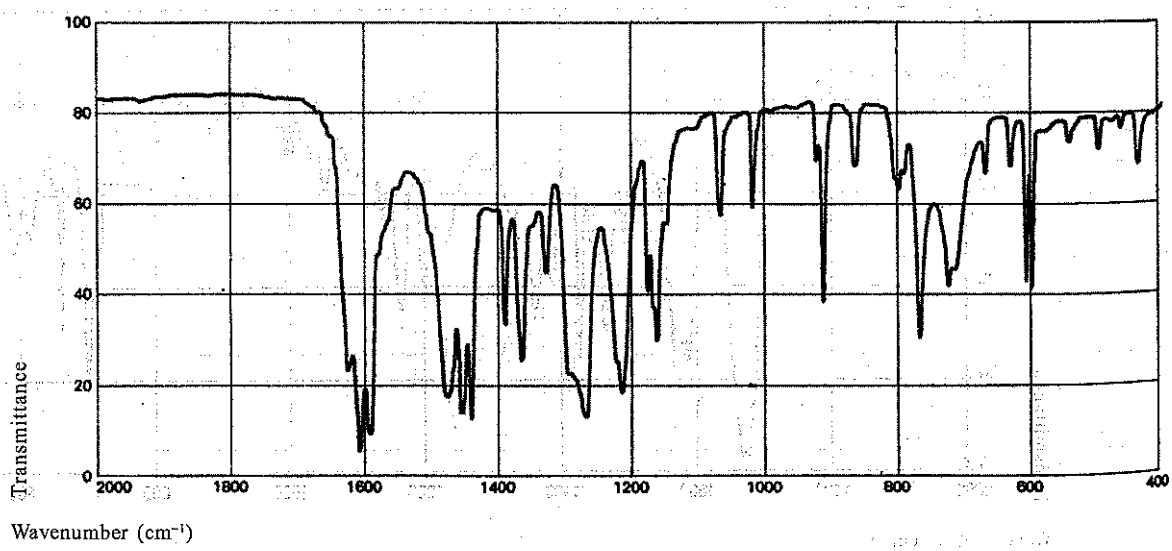
Disopyramide *Dispersive IR* Phase: 10 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



Disulfiram *Dispersive IR* Phase: KBr disc



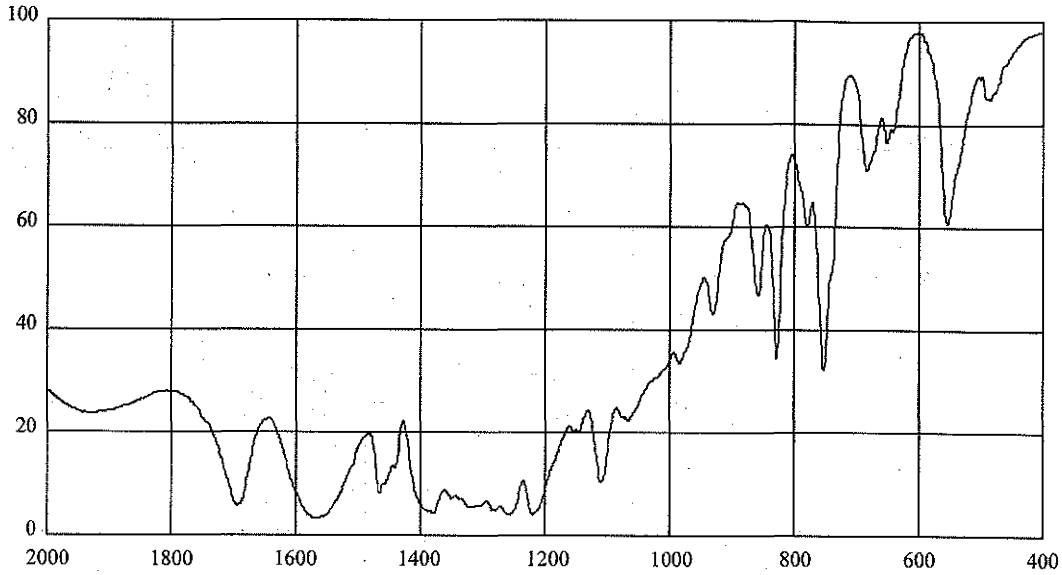
Dithranol *Dispersive IR* Phase: KBr disc



Divalproex Sodium

FTIR

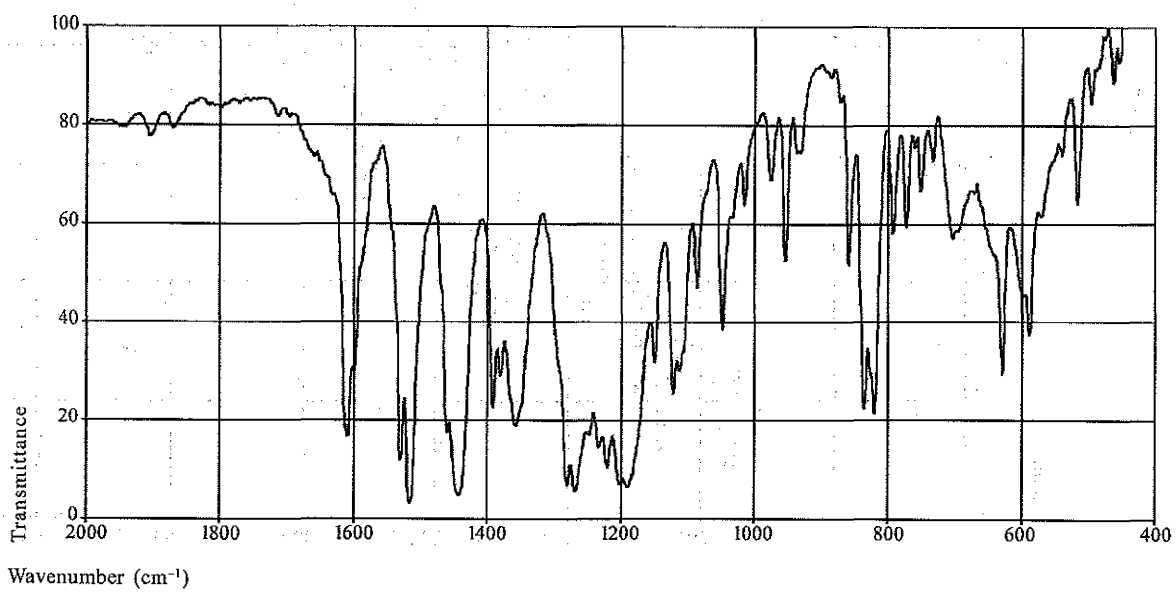
Phase: KBr disc



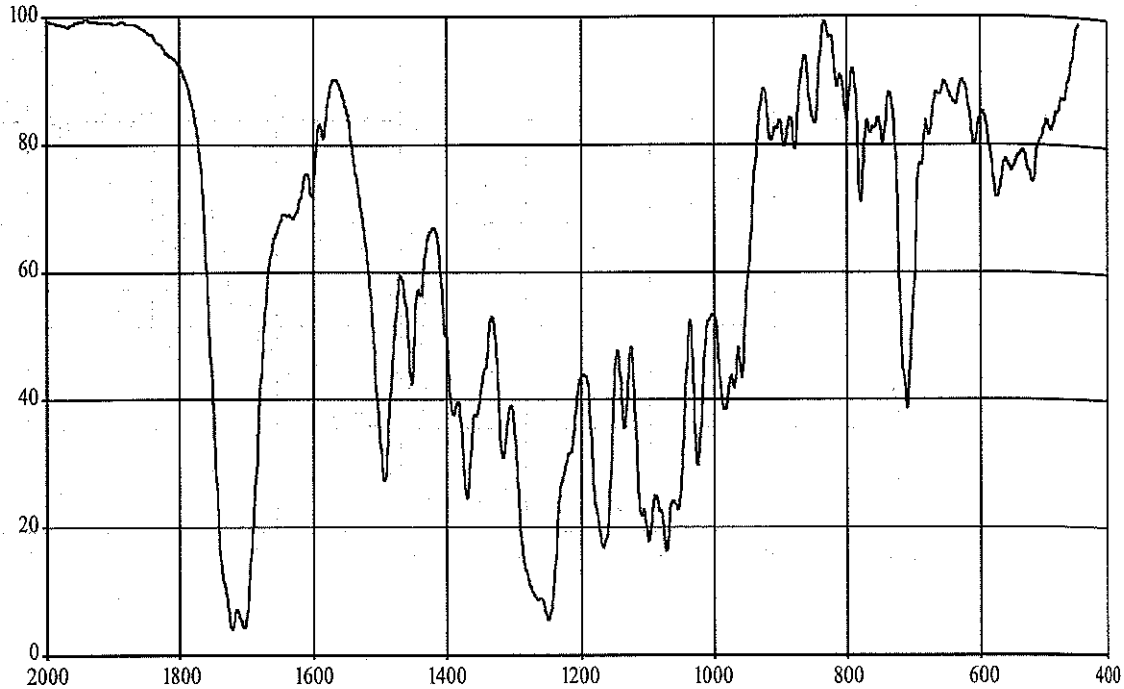
Dobutamine Hydrochloride

FTIR

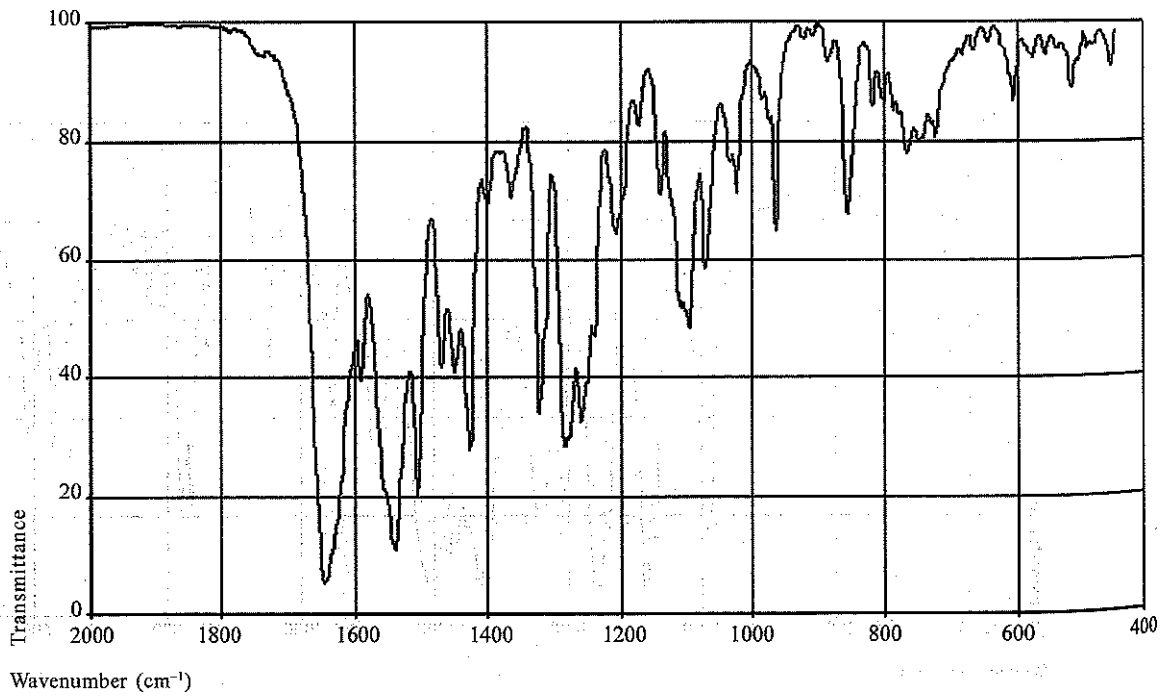
Phase: KBr disc



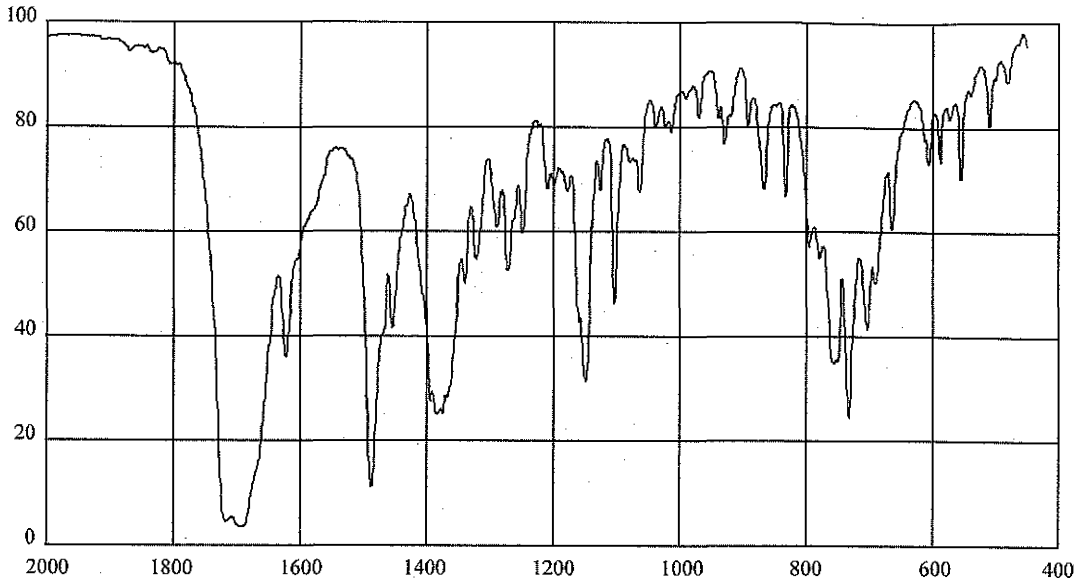
Docetaxel Anhydrous *FTIR* Phase: KBr disc



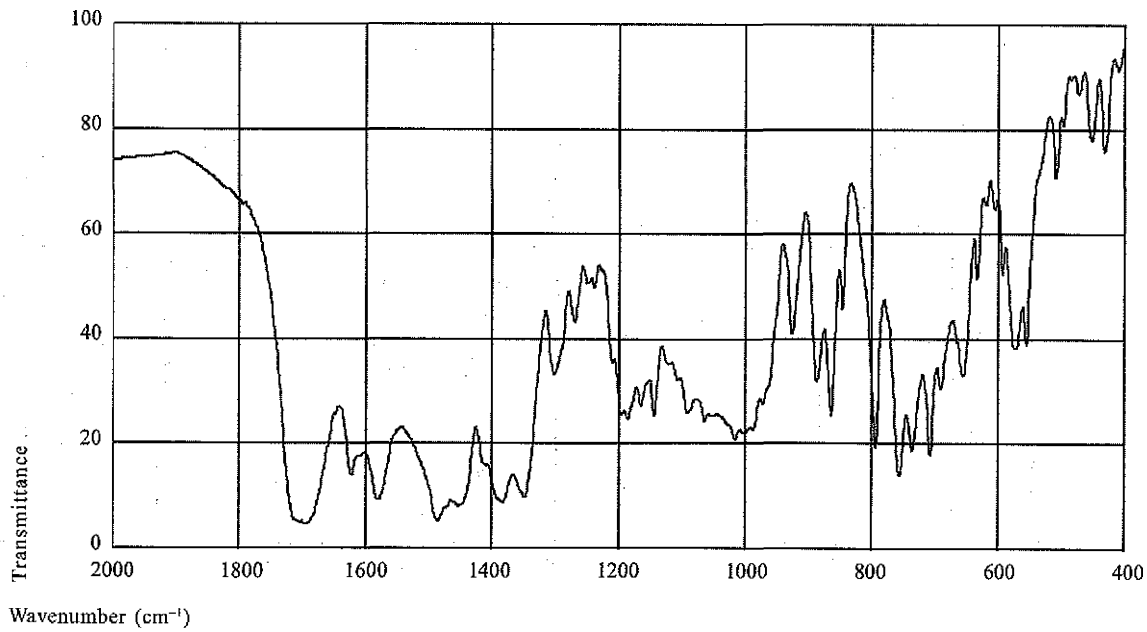
Dolutegravir Sodium *FTIR* Phase: KBr disc



Domperidone *FTIR* Phase: KBr disc



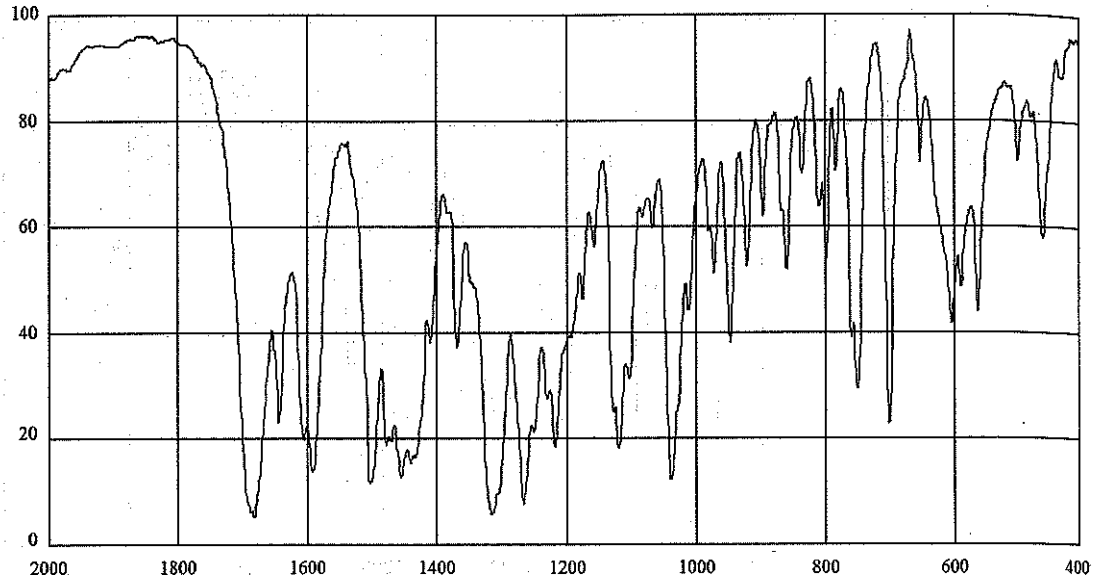
Domperidone Maleate *FTIR* Phase: KBr disc



Donepezil Hydrochloride

FTIR

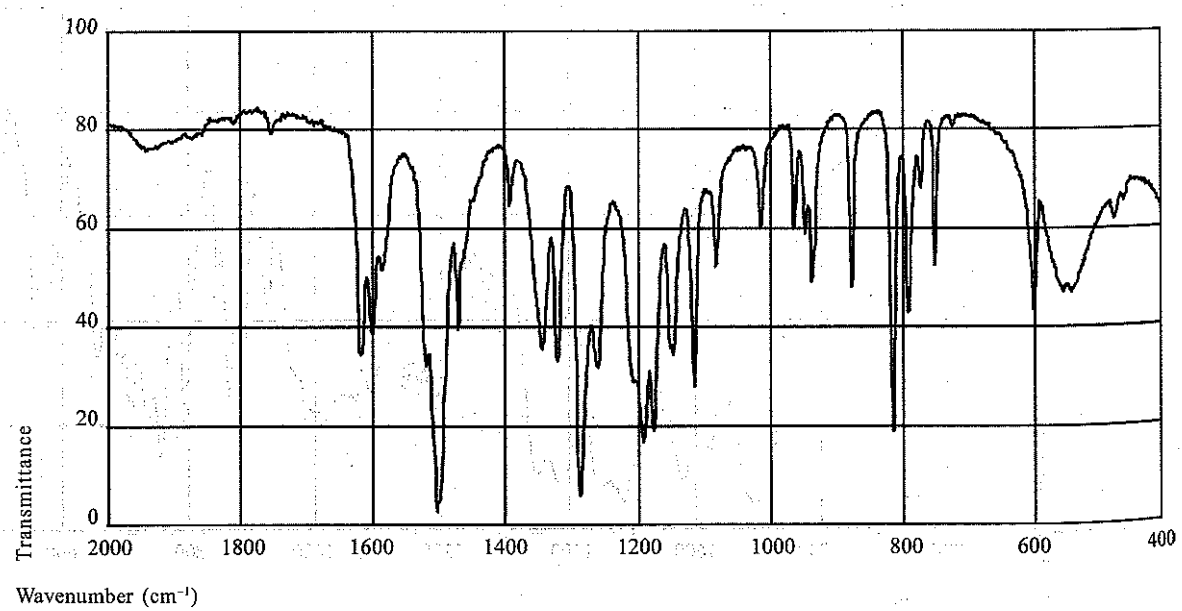
Phase: KBr disc



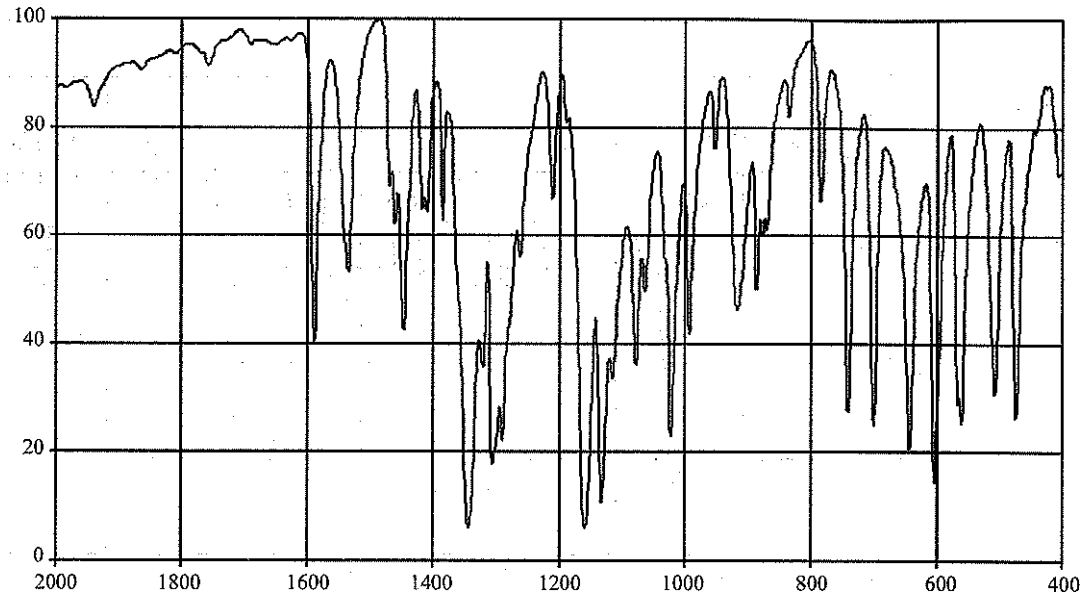
Dopamine Hydrochloride

FTIR

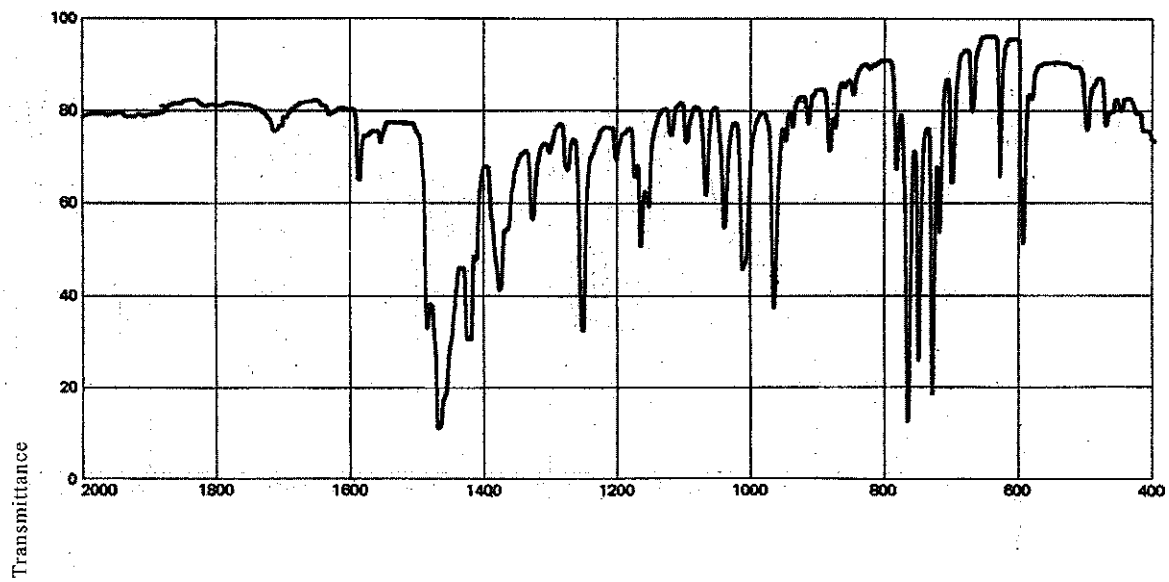
Phase: KBr disc



Dorzolamide Hydrochloride *FTIR* Phase: KBr disc



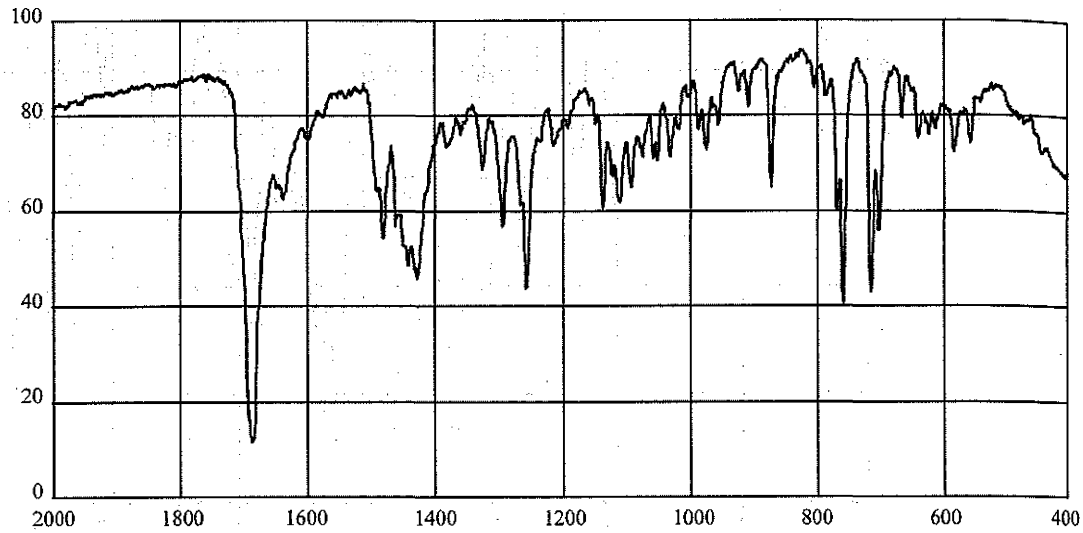
Dothiepin Hydrochloride *Dispersive IR* Phase: Liquid paraffin mull



Doxapram Hydrochloride

FTIR

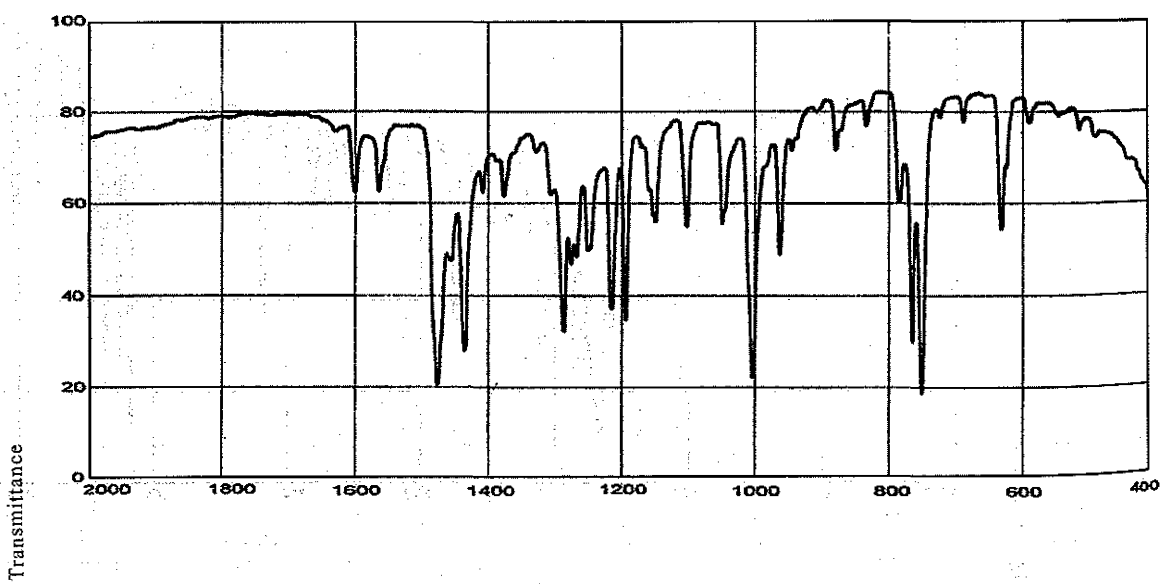
Phase: KBr disc



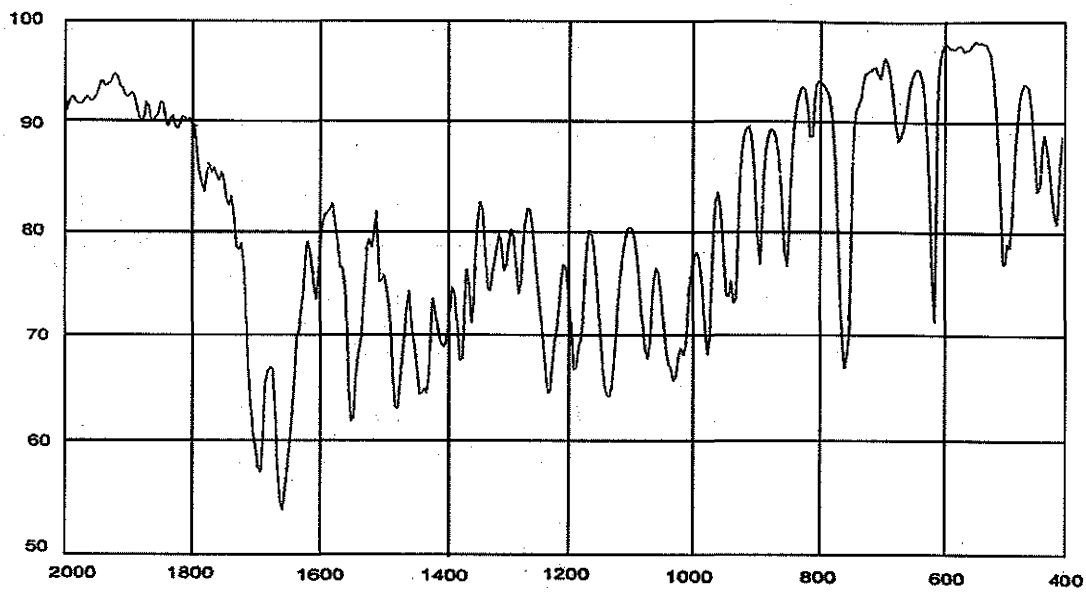
Doxepin Hydrochloride

Dispersive IR

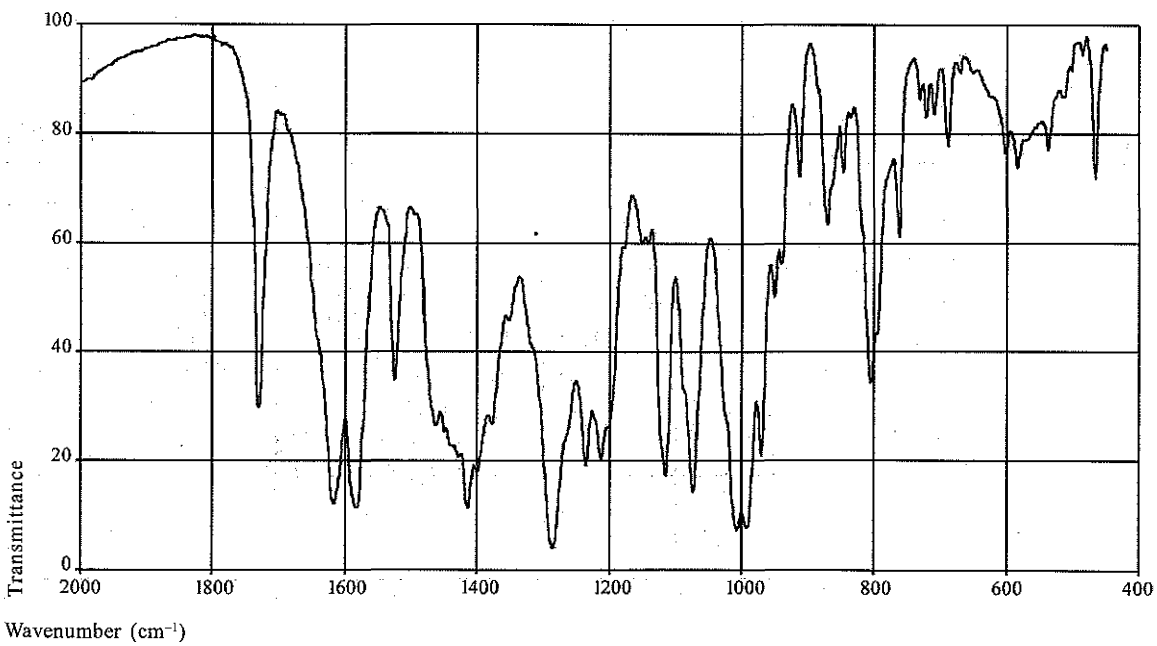
Phase: KCl disc



Doxofylline *Dispersive IR* Phase: KBr disc



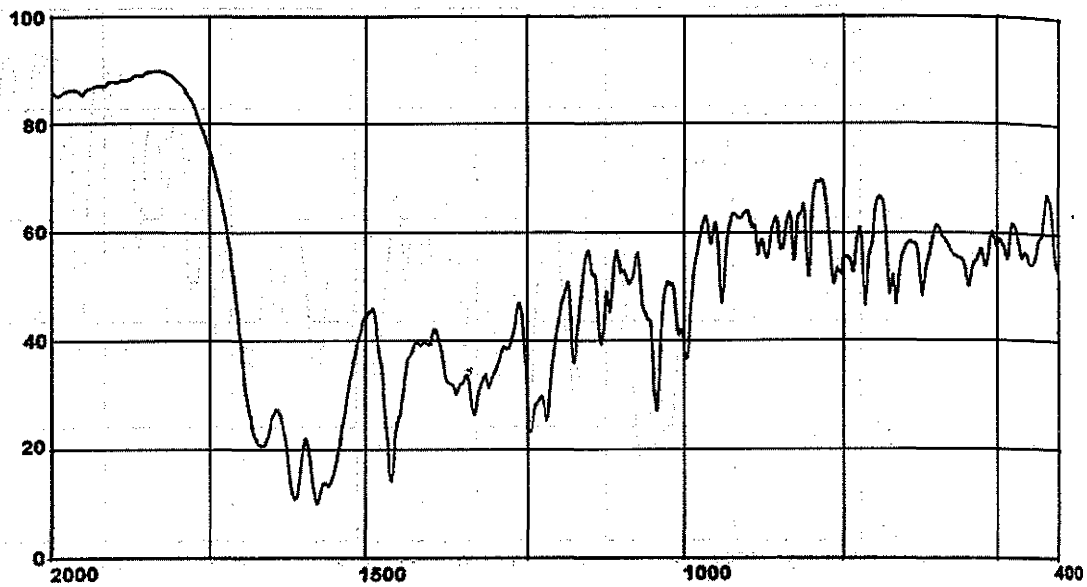
Doxorubicin Hydrochloride *FTIR* Phase: KBr disc



Doxycycline Hydrochloride

FTIR

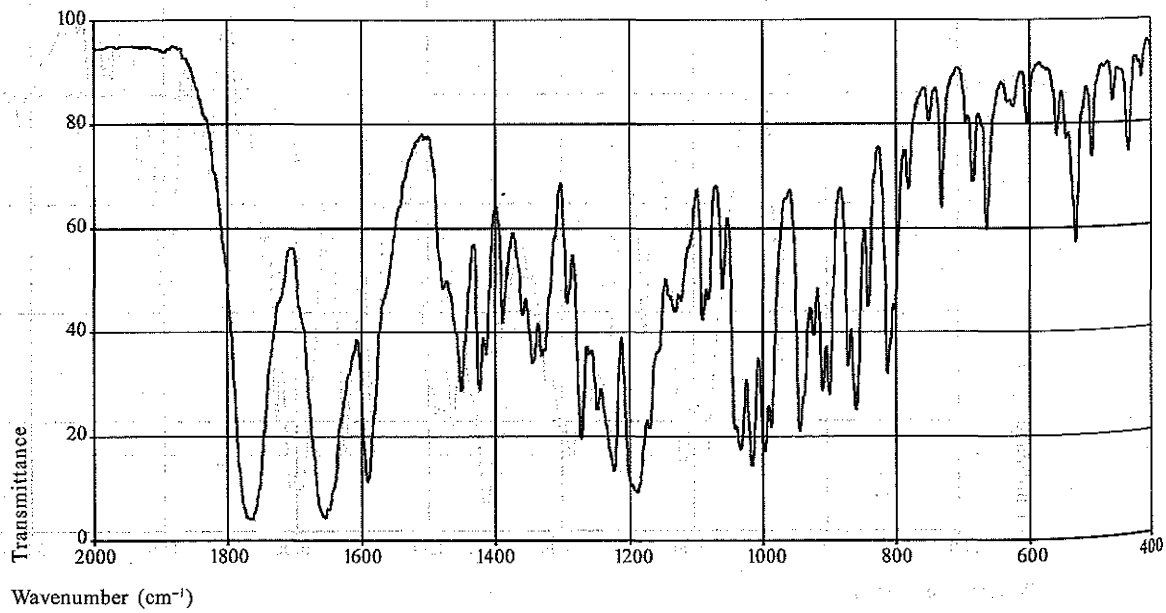
Phase: KBr disc



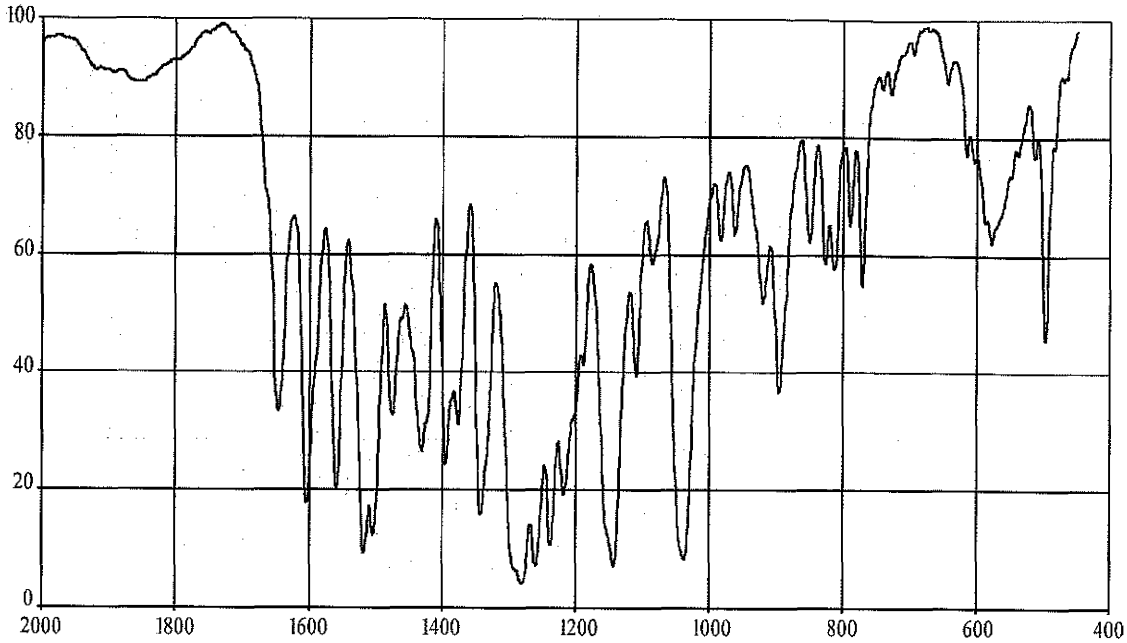
Drospirenone

FTIR

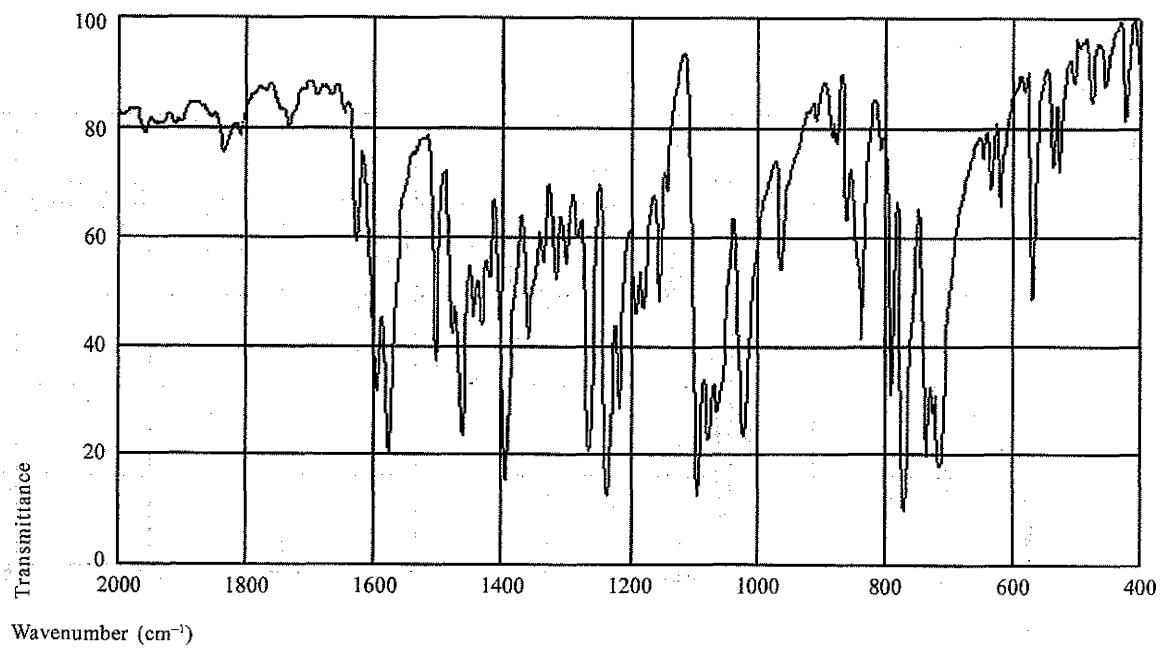
Phase: KBr disc



Drotaverine Hydrochloride *FTIR* Phase: KBr disc



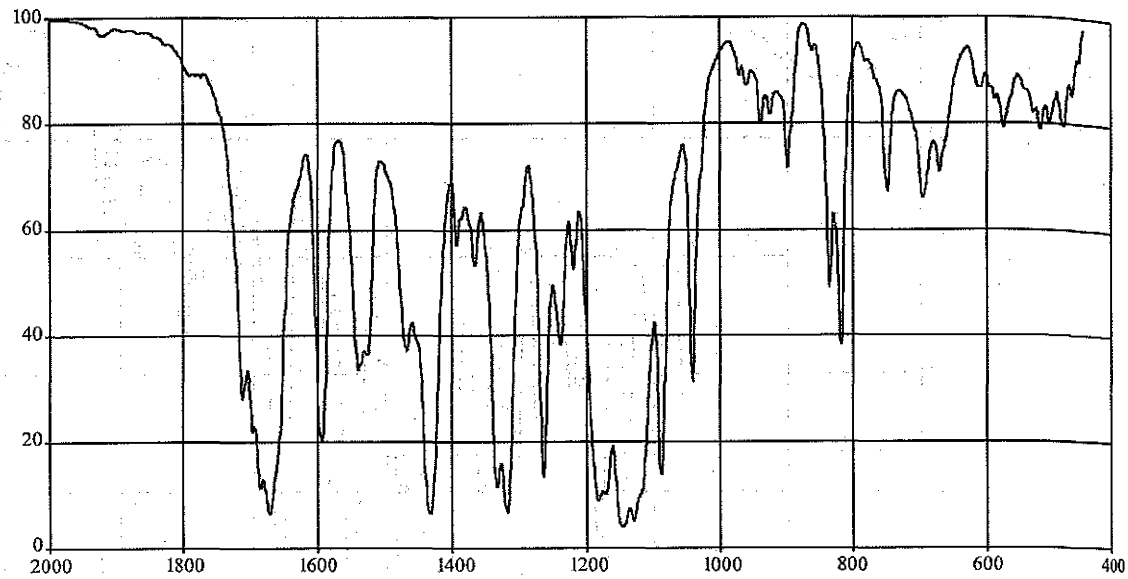
Duloxetine Hydrochloride *FTIR* Phase: KBr disc



Dutasteride

FTIR

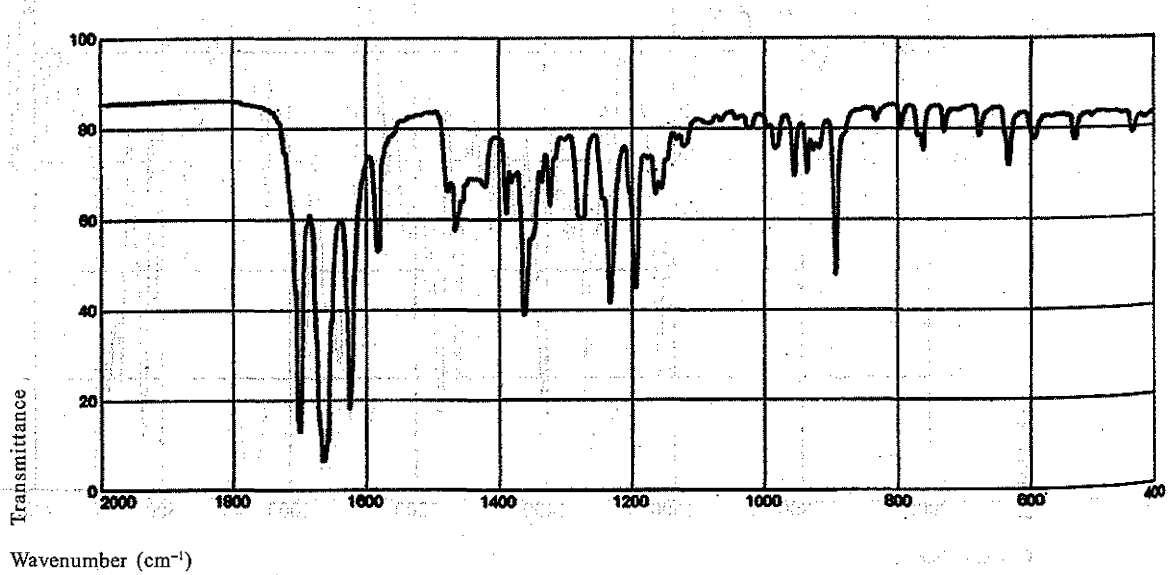
Phase: KBr disc



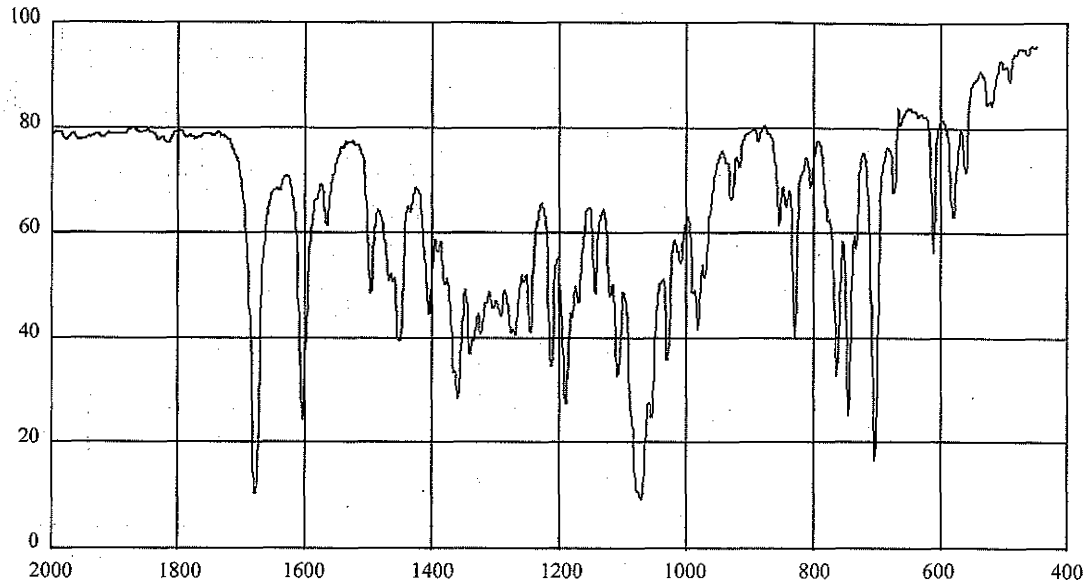
Dydrogesterone

Dispersive IR

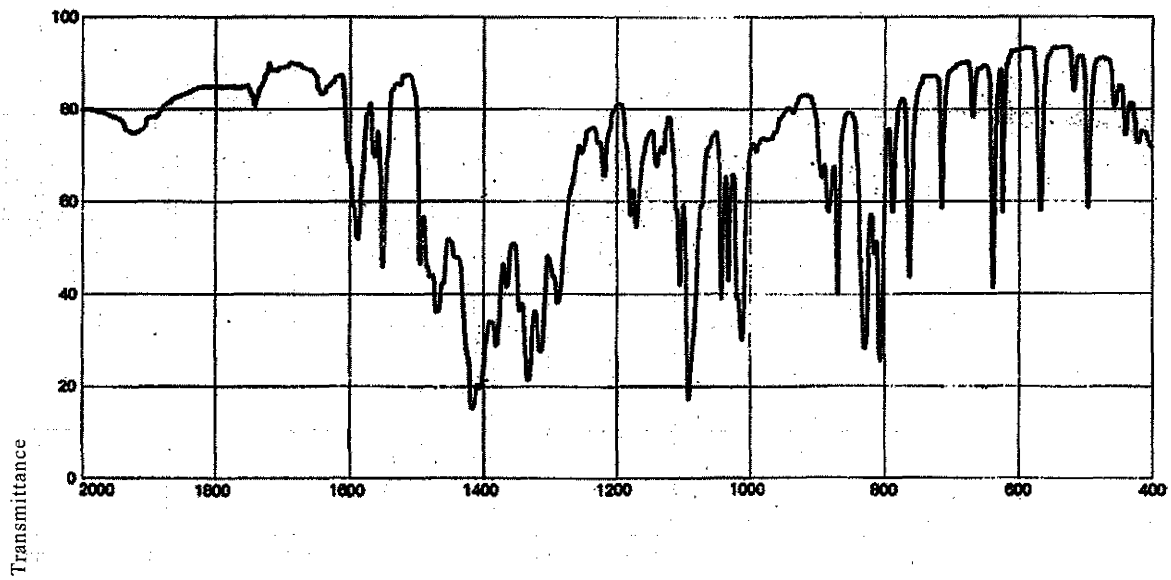
Phase: KBr disc



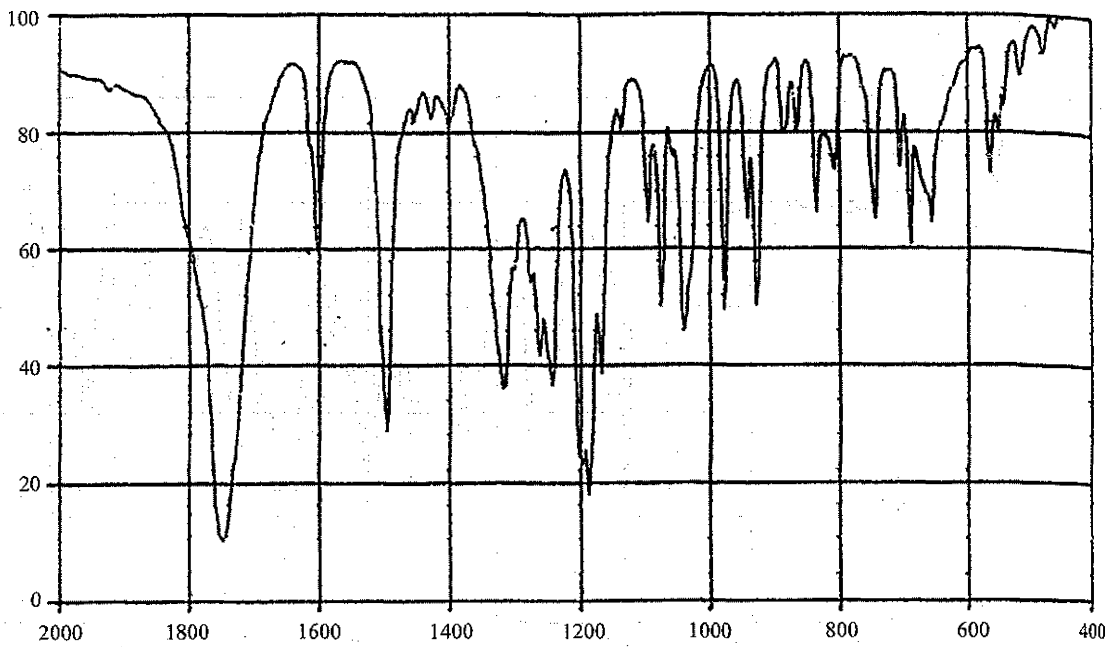
Ebastine *FTIR* Phase: KBr disc



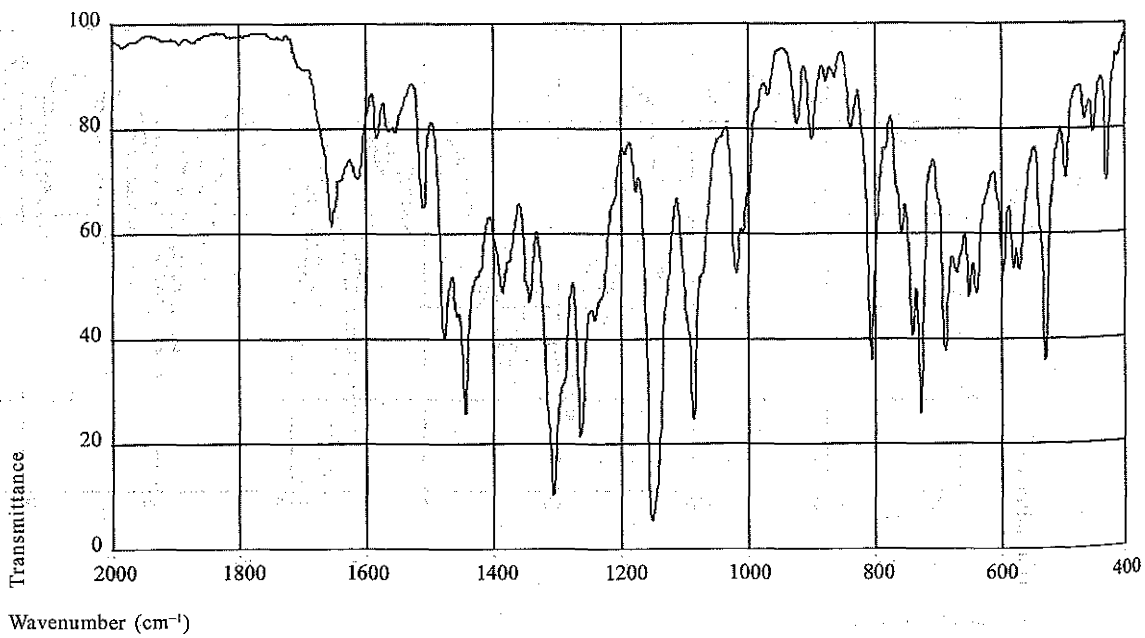
Econazole Nitrate *Dispersive IR* Phase: Liquid paraffin mull



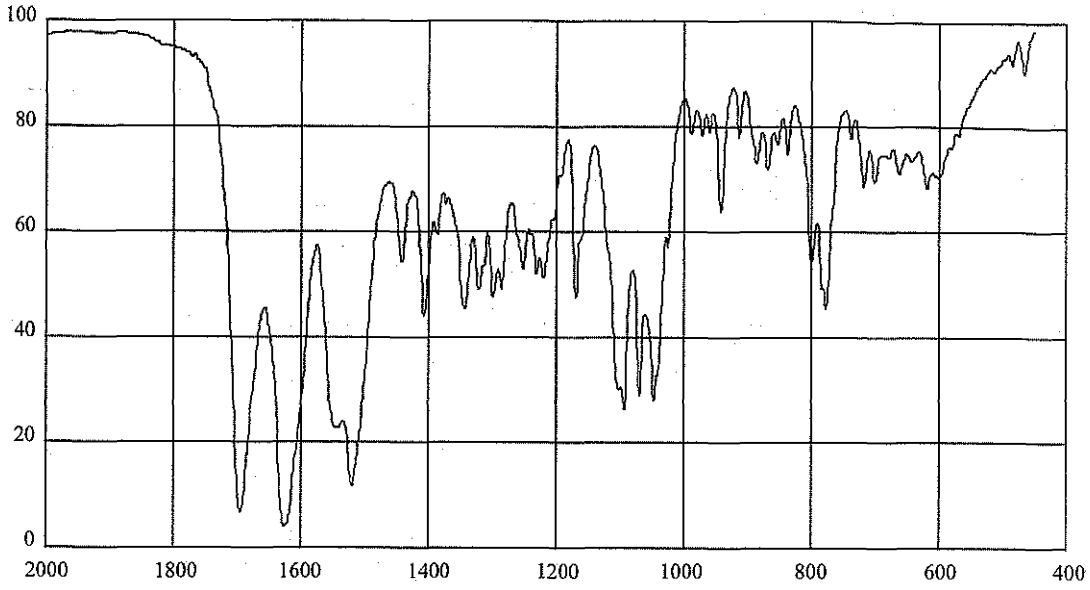
Efavirenz *FTIR* Phase: KBr disc



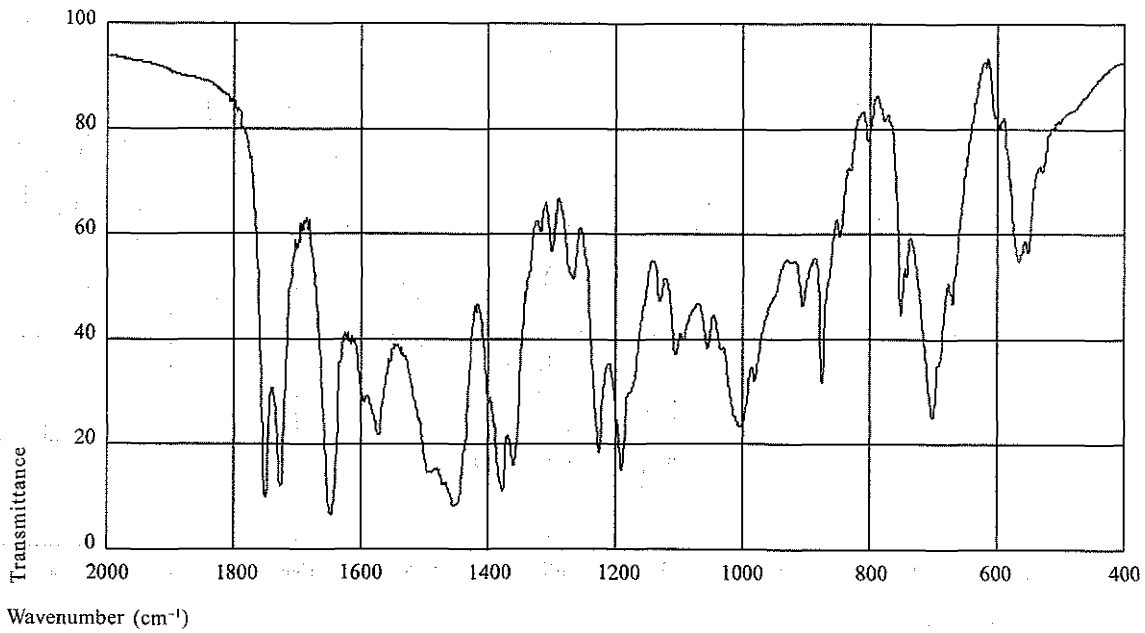
Eletriptan Hydrobromide *FTIR* Phase: KBr disc



Emtricitabine *FTIR* Phase: KBr disc



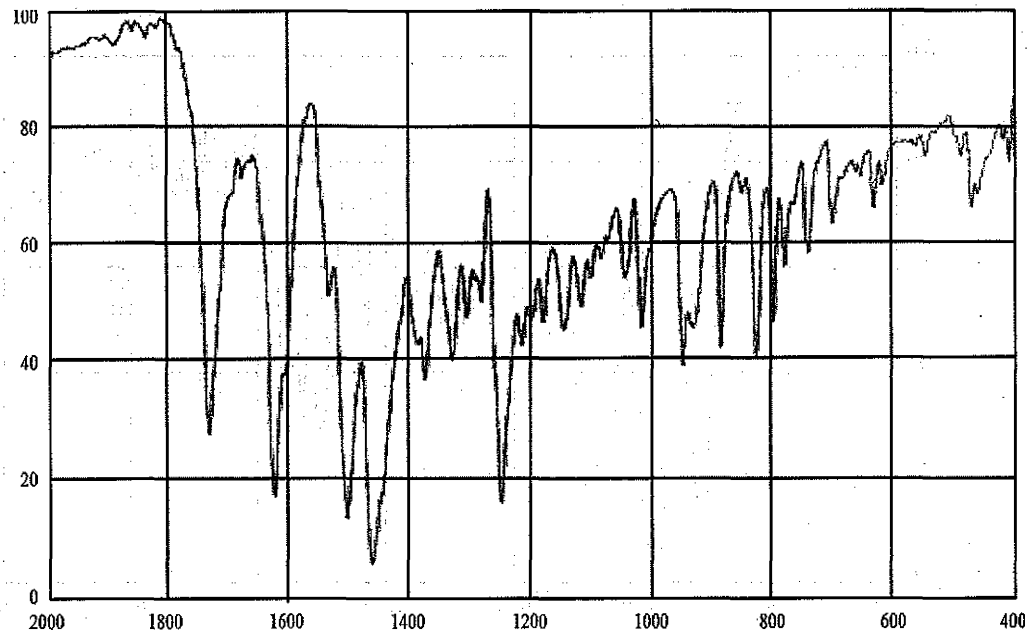
Enalapril Maleate *FTIR* Phase: KBr disc



Enrofloxacin

FTIR

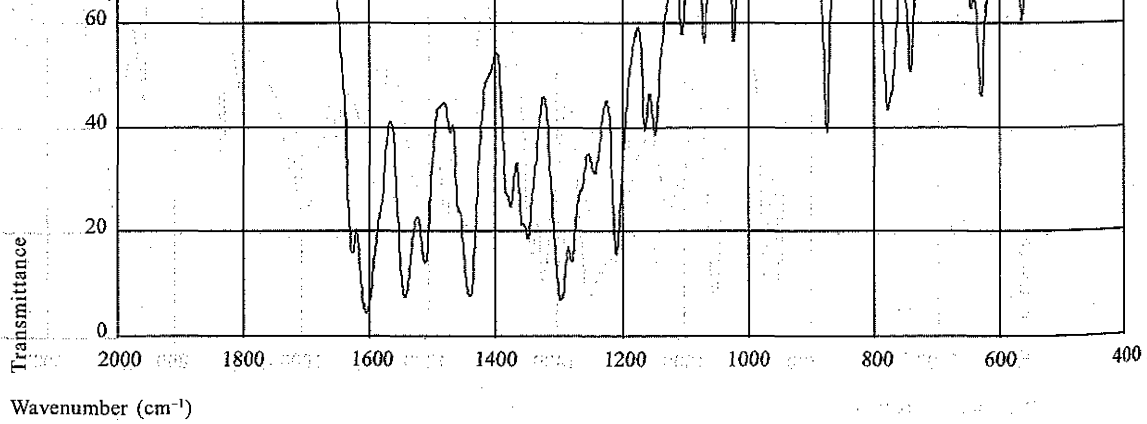
Phase: KBr disc



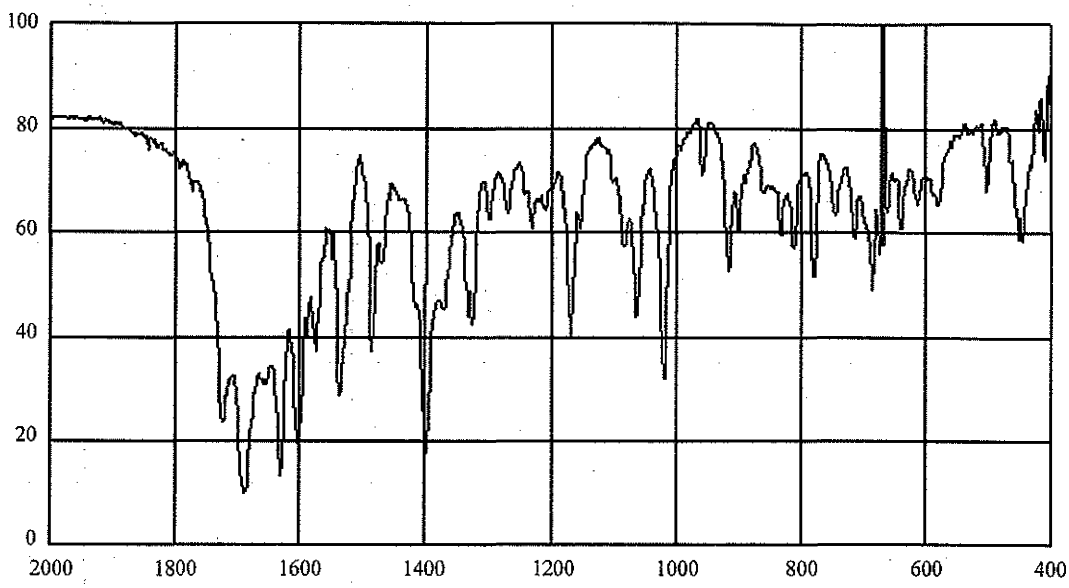
Entacapone

FTIR

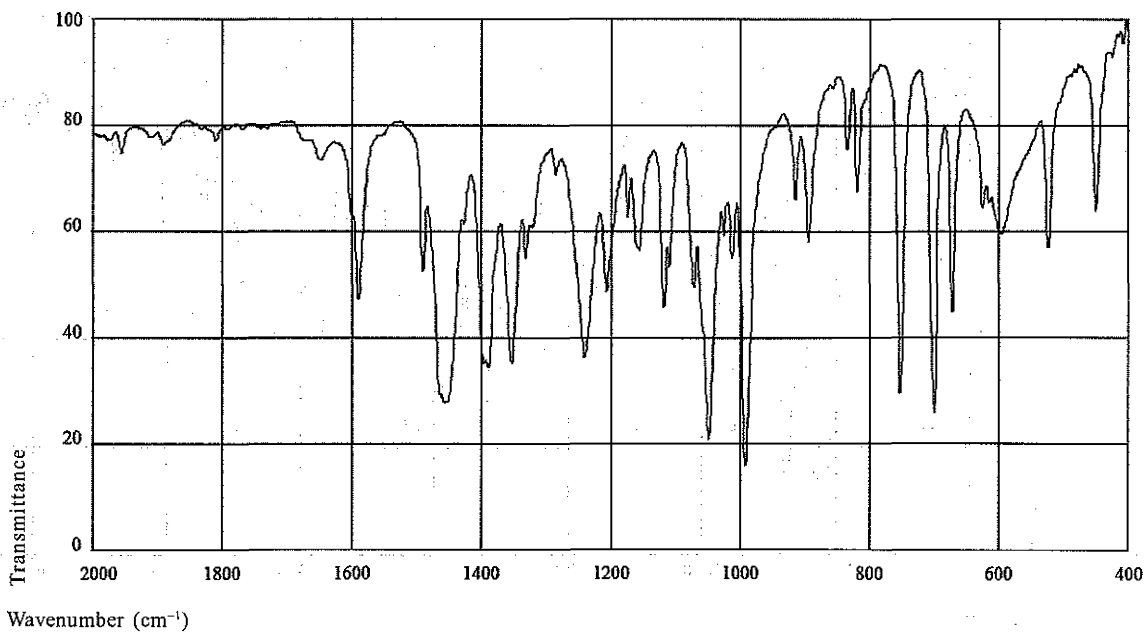
Phase: KBr disc



Entecavir Monohydrate *FTIR* Phase: KBr disc



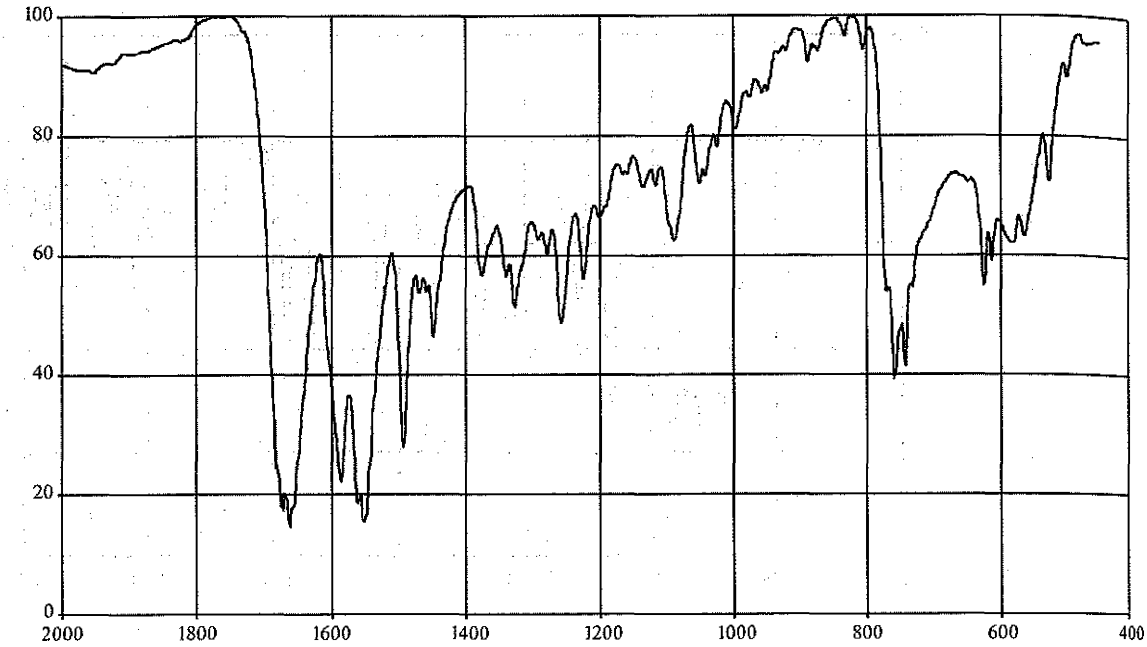
Ephedrine Hydrochloride *FTIR* Phase: KBr disc



Epinastine Hydrochloride

FTIR

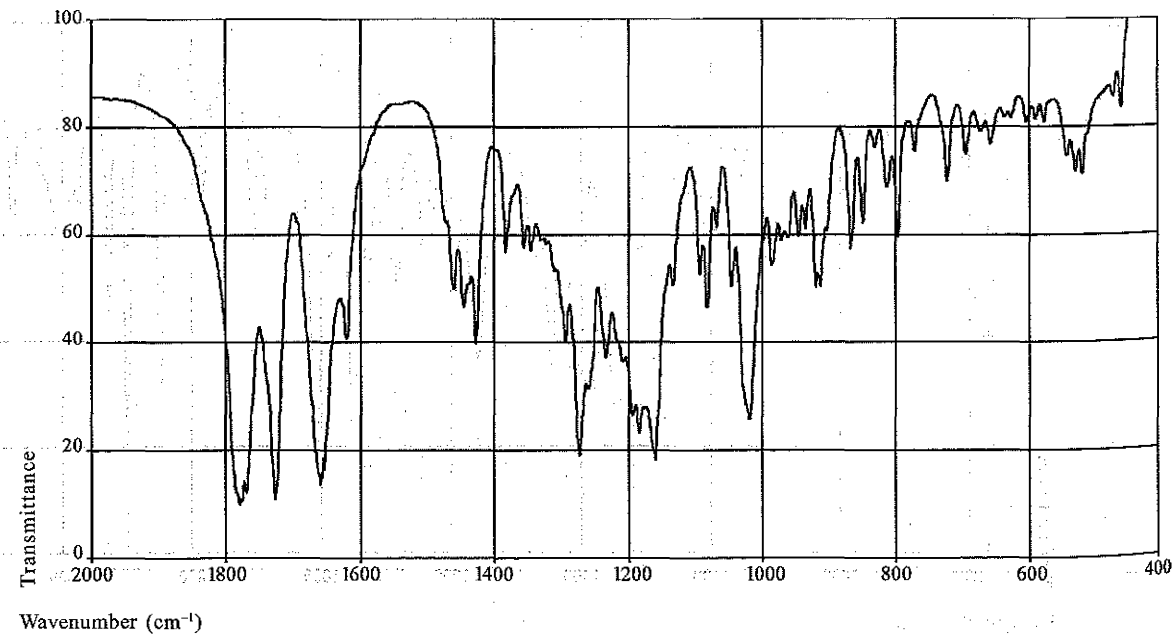
Phase: KBr disc



Eplerenone

FTIR

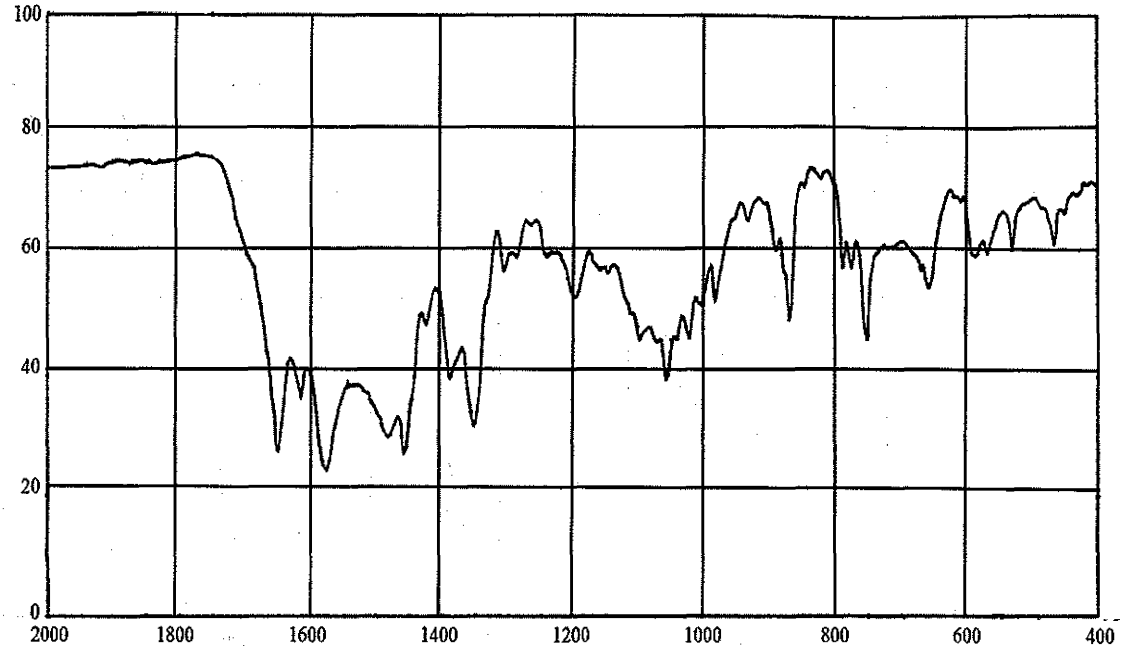
Phase: KBr disc



Ergometrine Maleate

FTIR

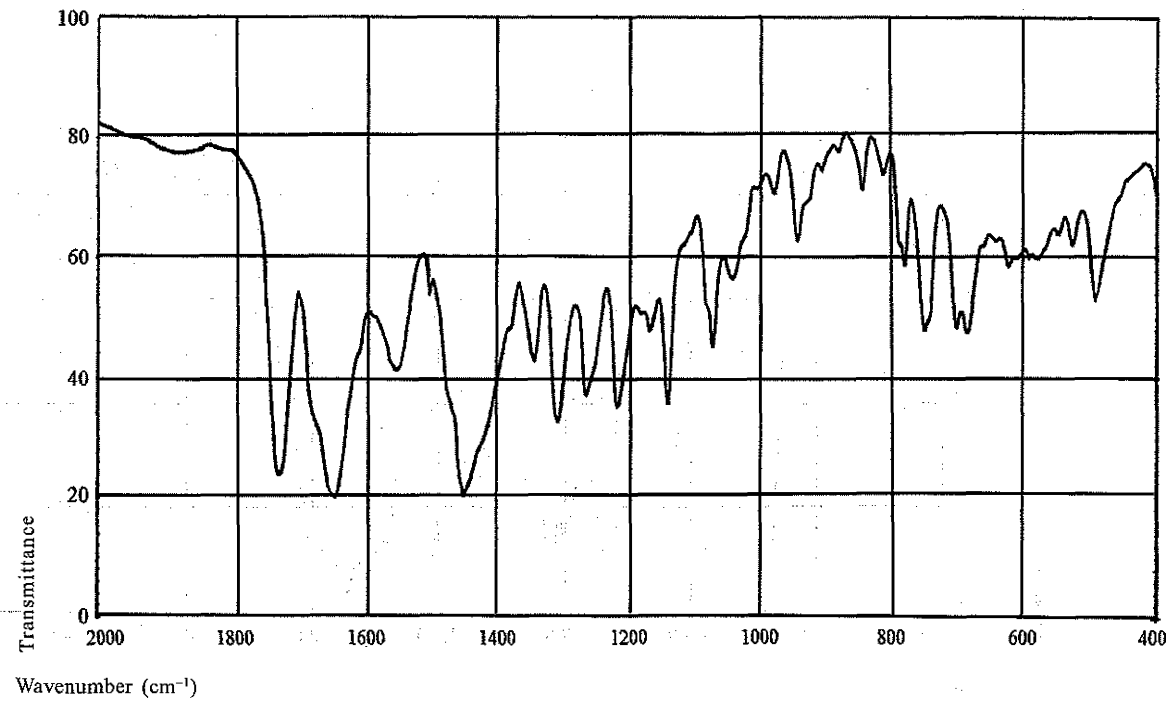
Phase: KBr disc



Ergotamine Tartrate

FTIR

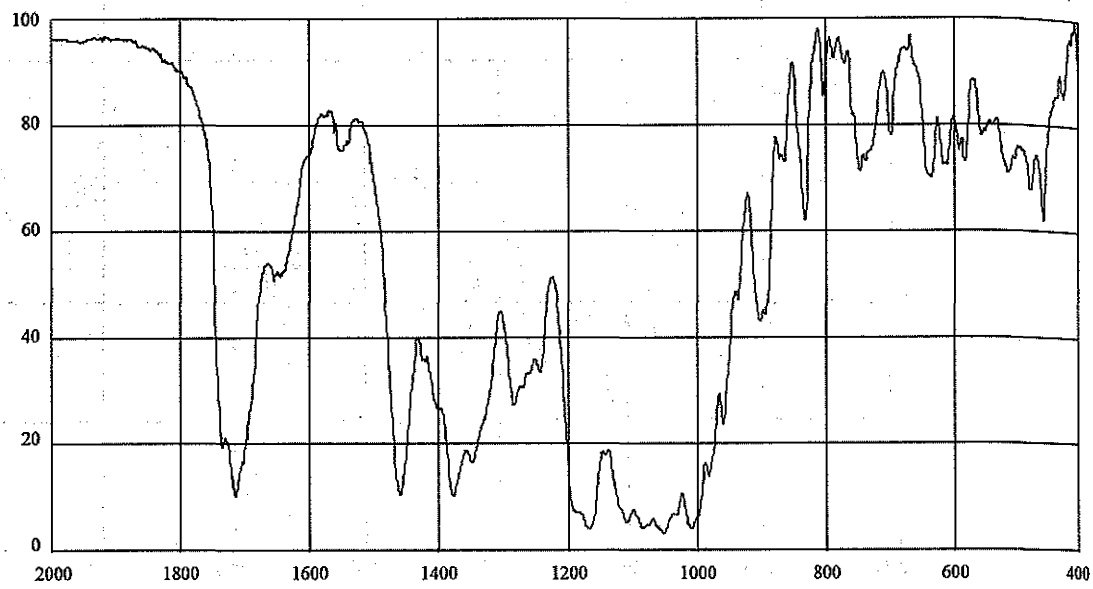
Phase: KBr disc



Erythromycin

FTIR

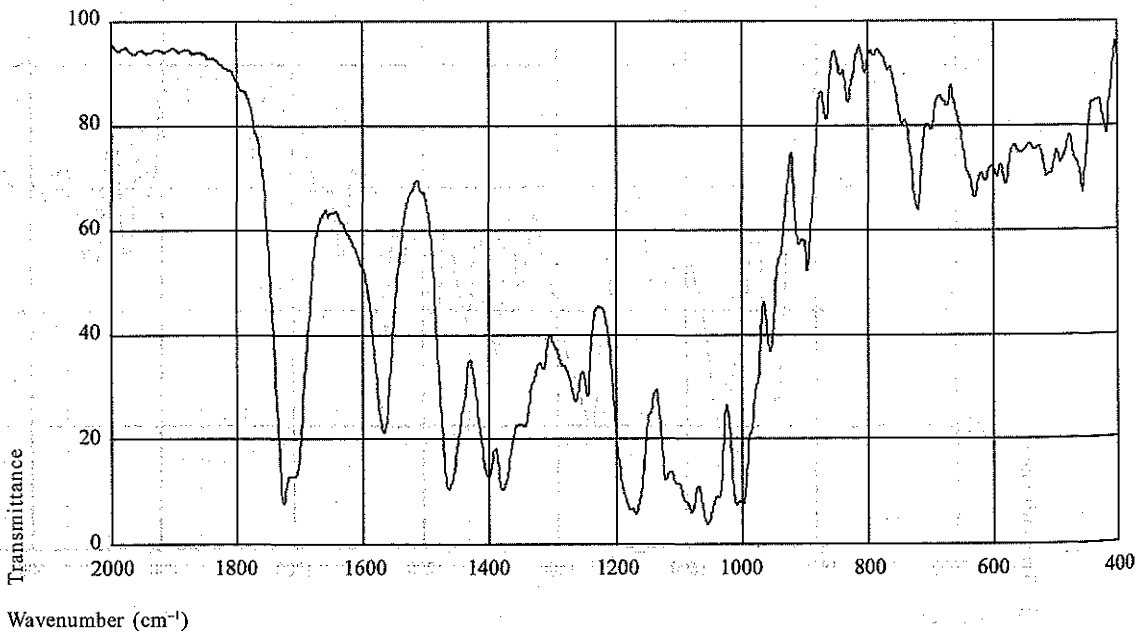
Phase: KBr disc



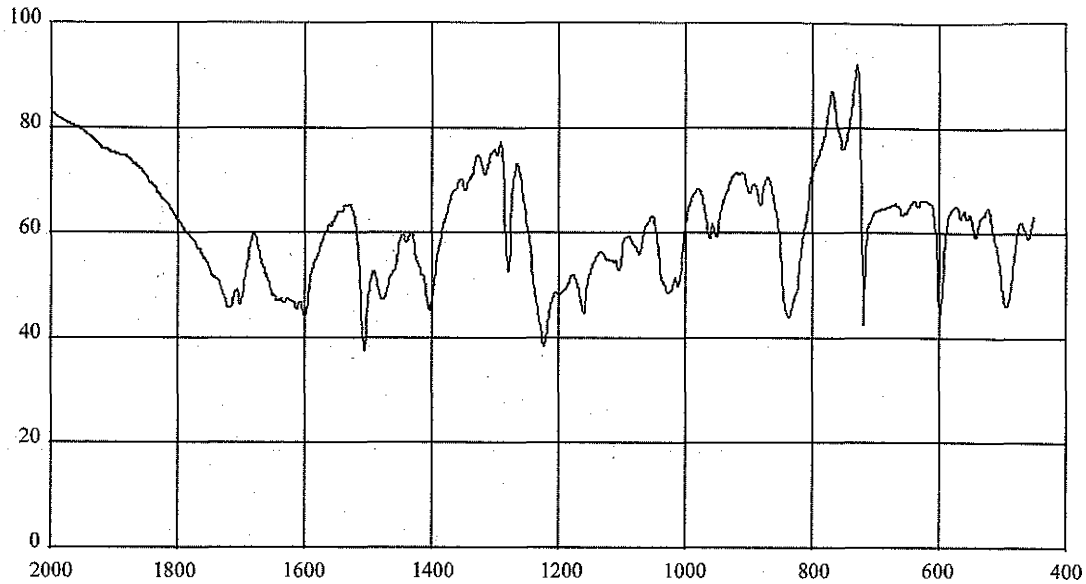
Erythromycin Stearate

FTIR

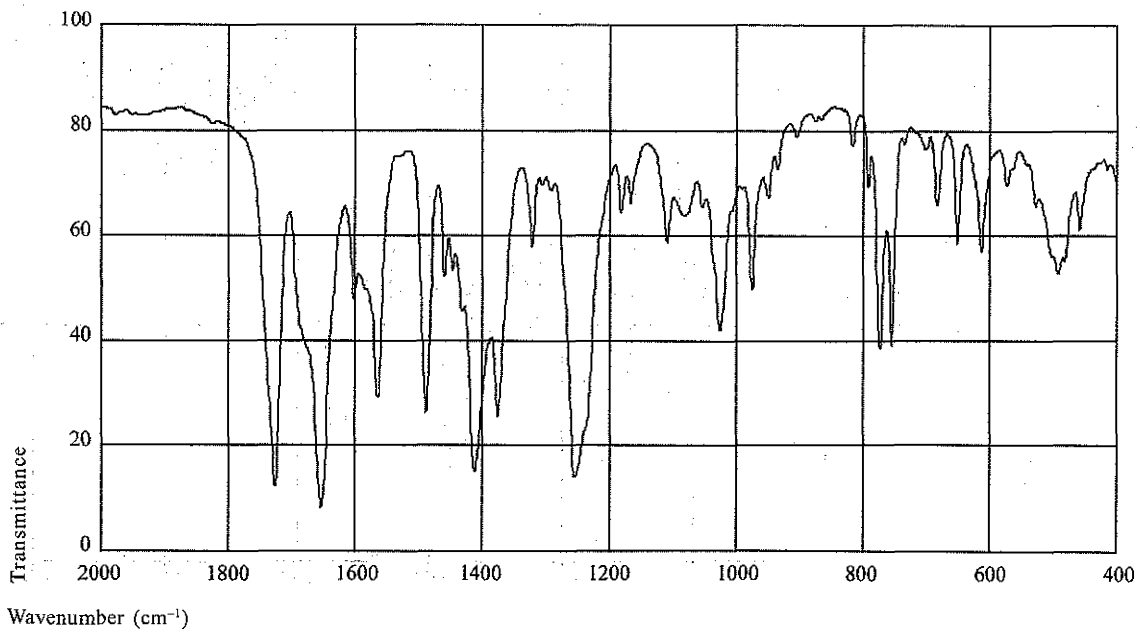
Phase: KBr disc



Escitalopram Oxalate *FTIR* Phase: KBr disc



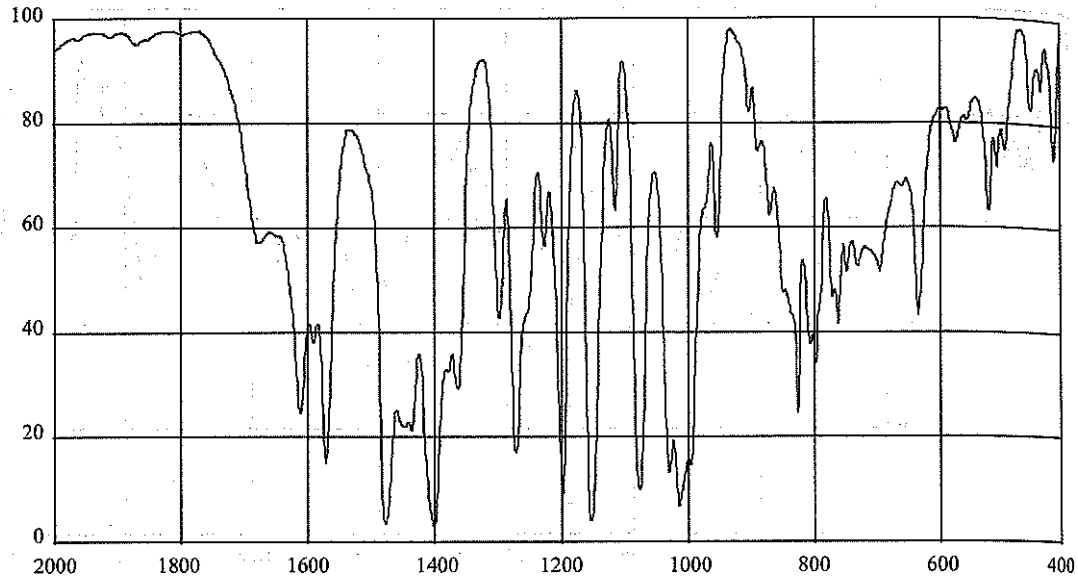
Eslicarbazepine Acetate *FTIR* Phase: KBr disc



Esomeprazole Magnesium

FTIR

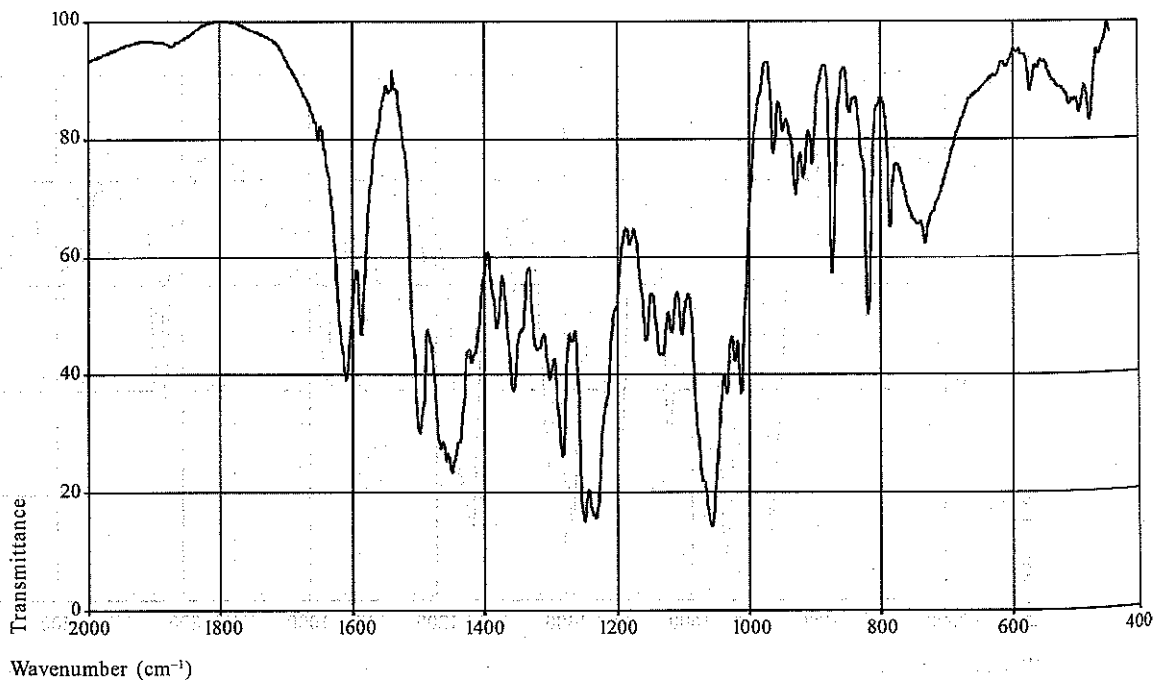
Phase: KBr disc



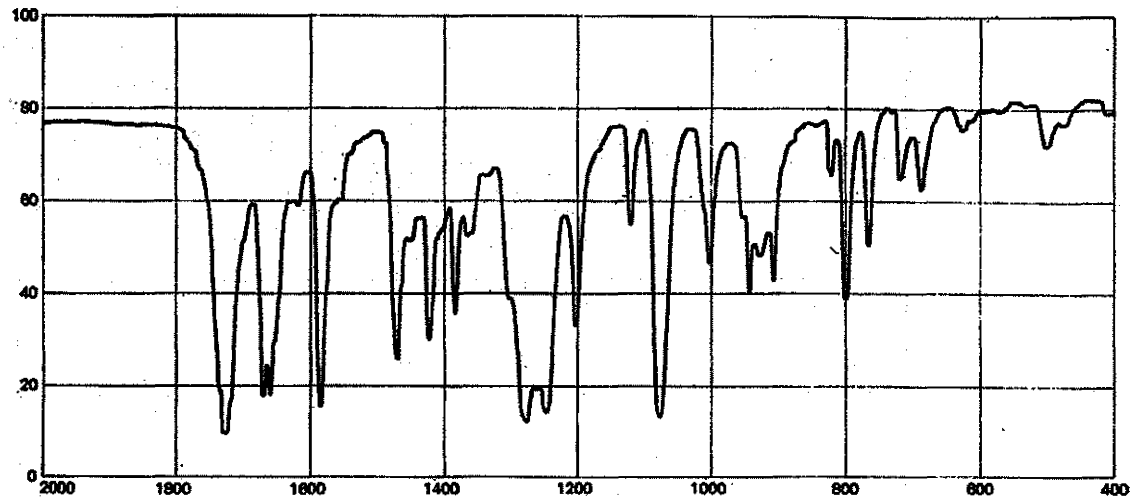
Estradiol Hemihydrate

FTIR

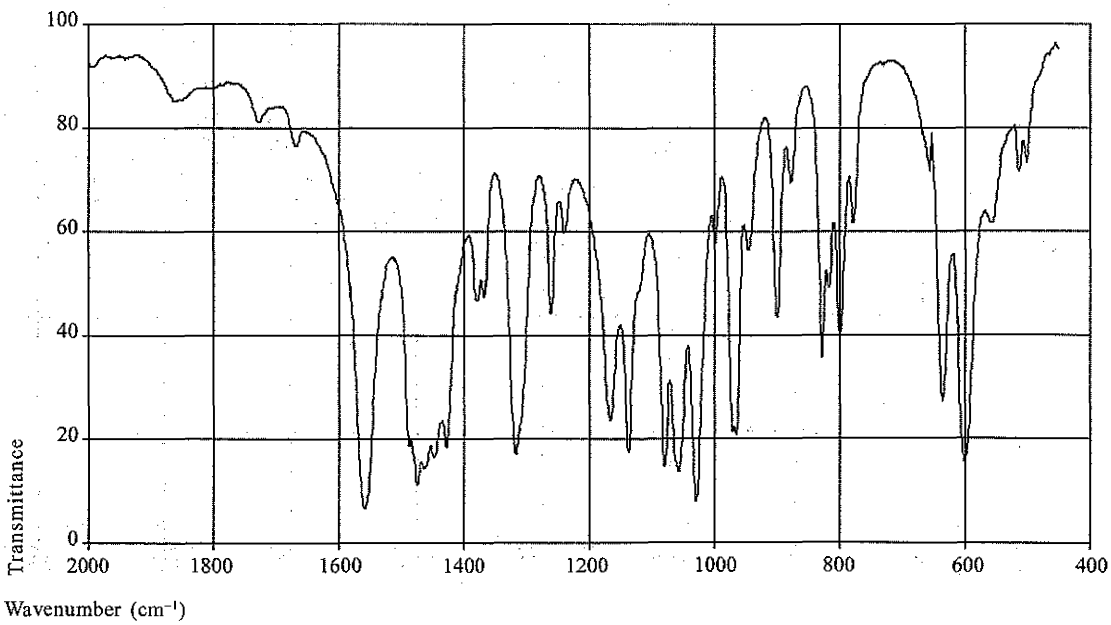
Phase: KBr disc



Ethacrynic Acid *Dispersive IR* Phase: KBr disc



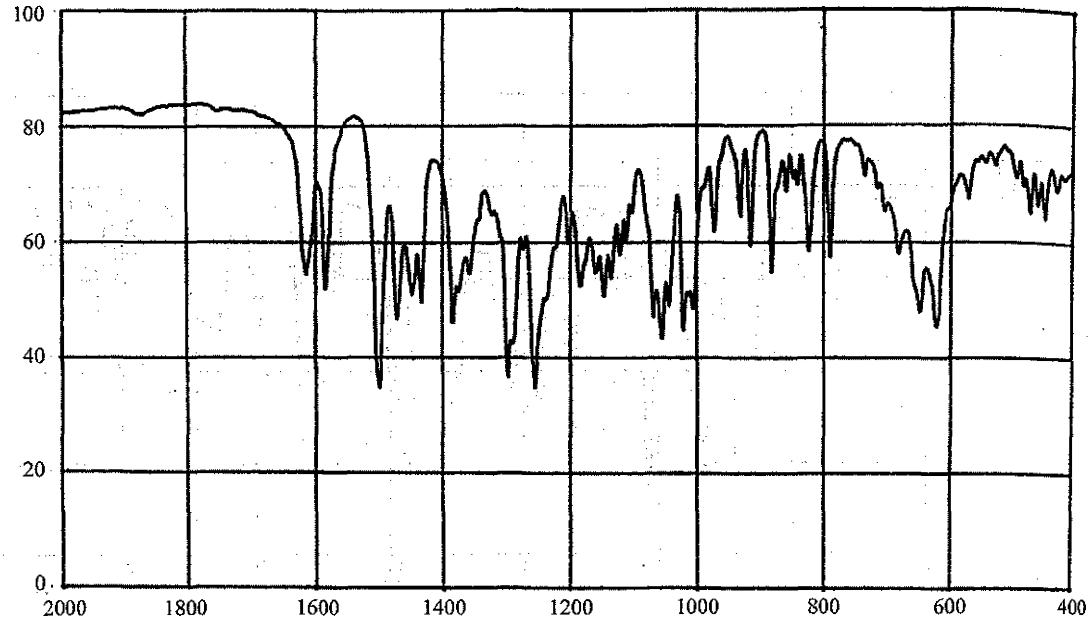
Ethambutol Hydrochloride *FTIR* Phase: KBr disc



Ethinylestradiol

FTIR

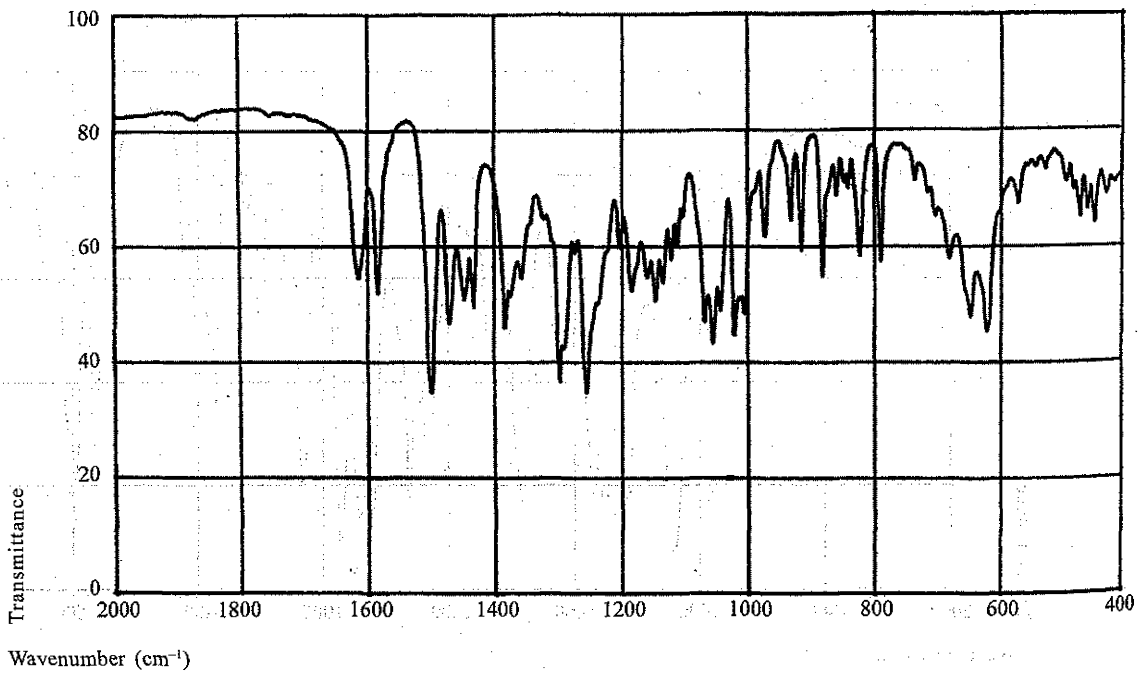
Phase: KBr disc



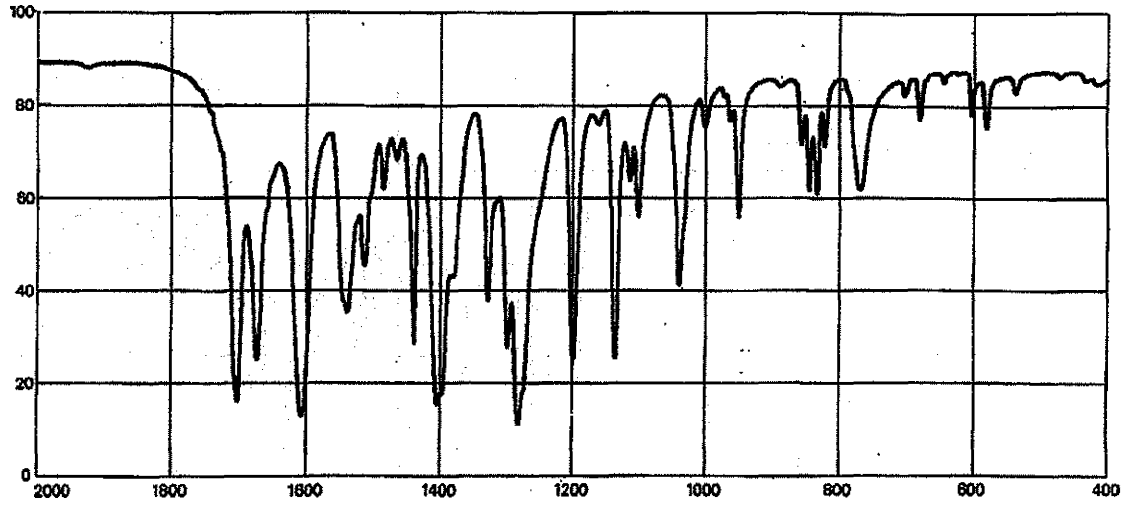
Ethionamide

FTIR

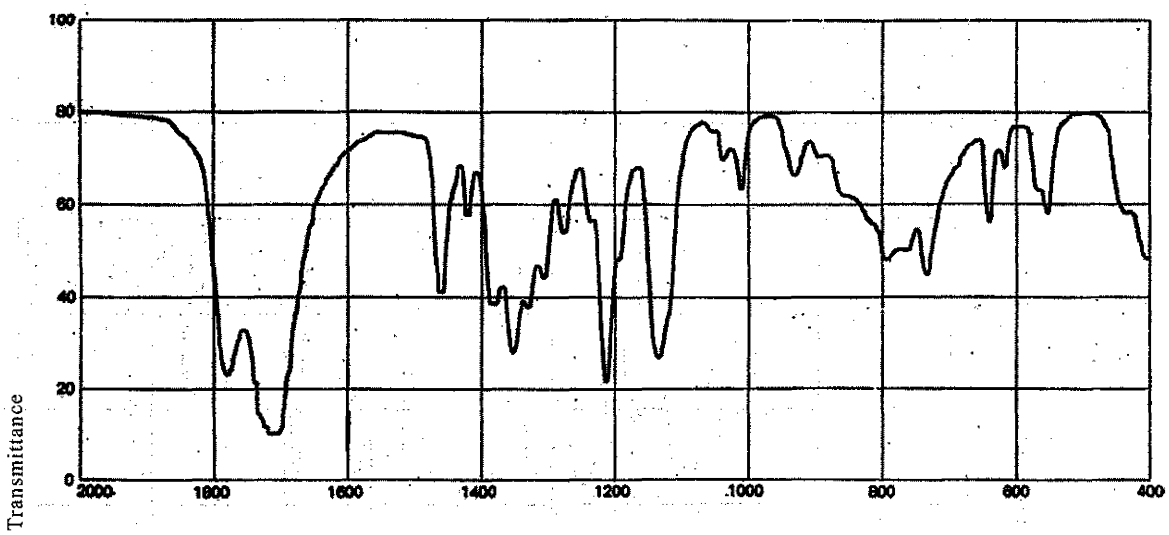
Phase: KBr disc



Ethopabate *Dispersive IR* Phase: KBr disc



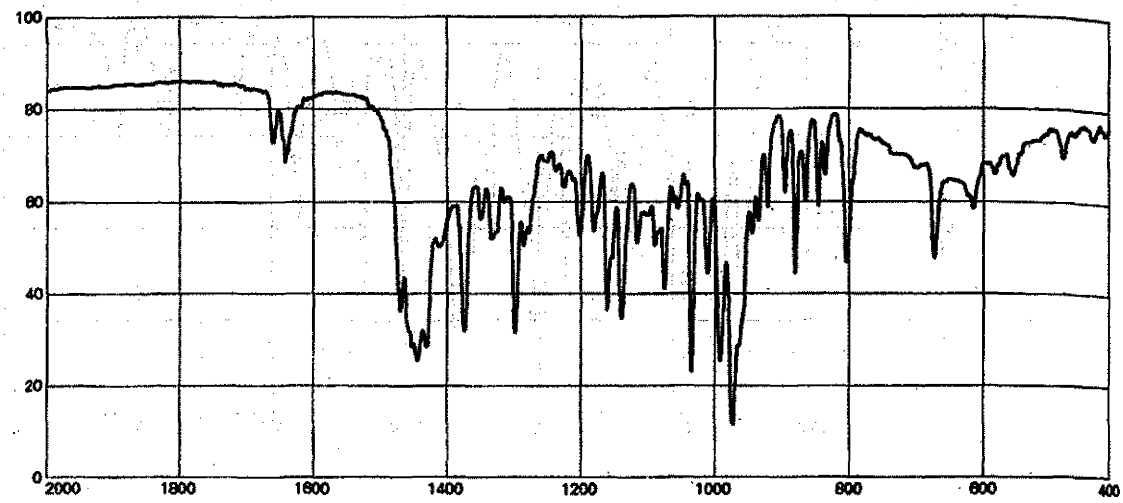
Ethosuximide *FTIR* Phase: Thin film



Ethyloestrenol

Dispersive IR

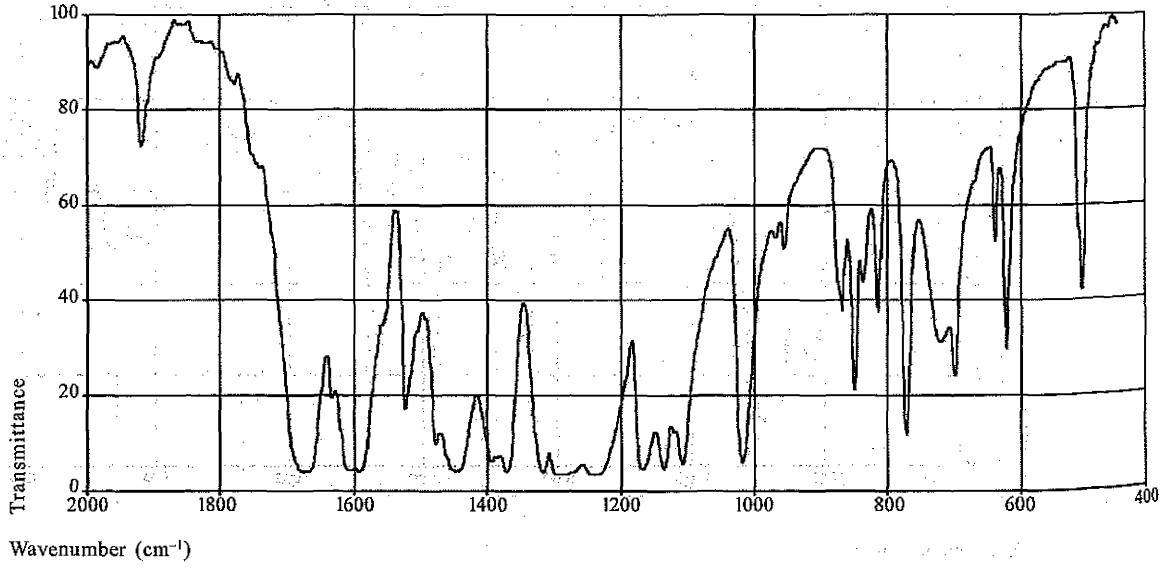
Phase: KBr disc



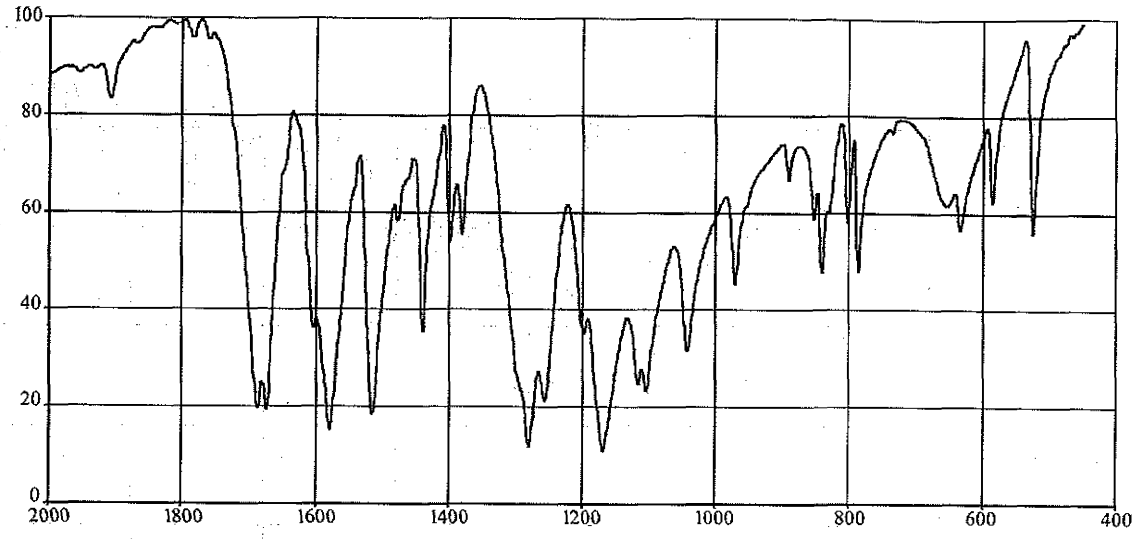
Ethylparaben

FTIR

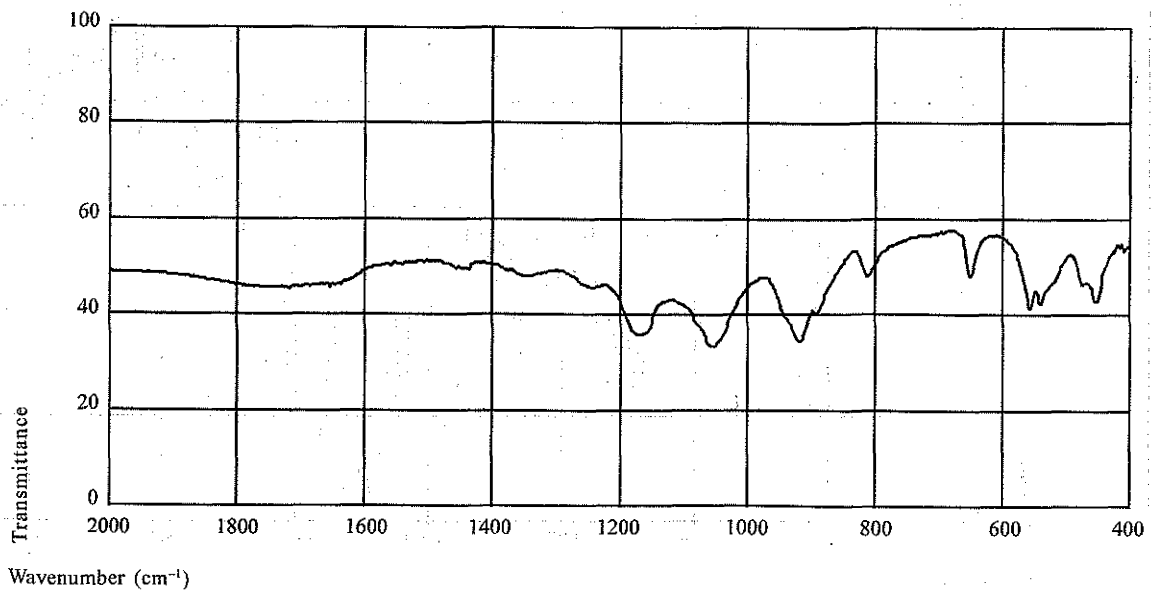
Phase: KBr disc



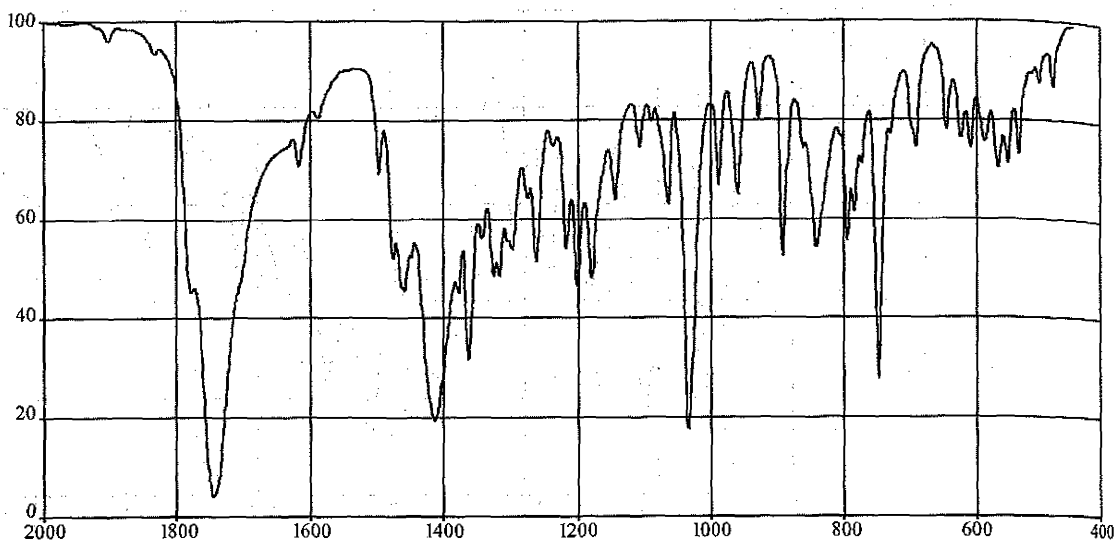
Ethyl Vanillin *FTIR* Phase: KBr disc



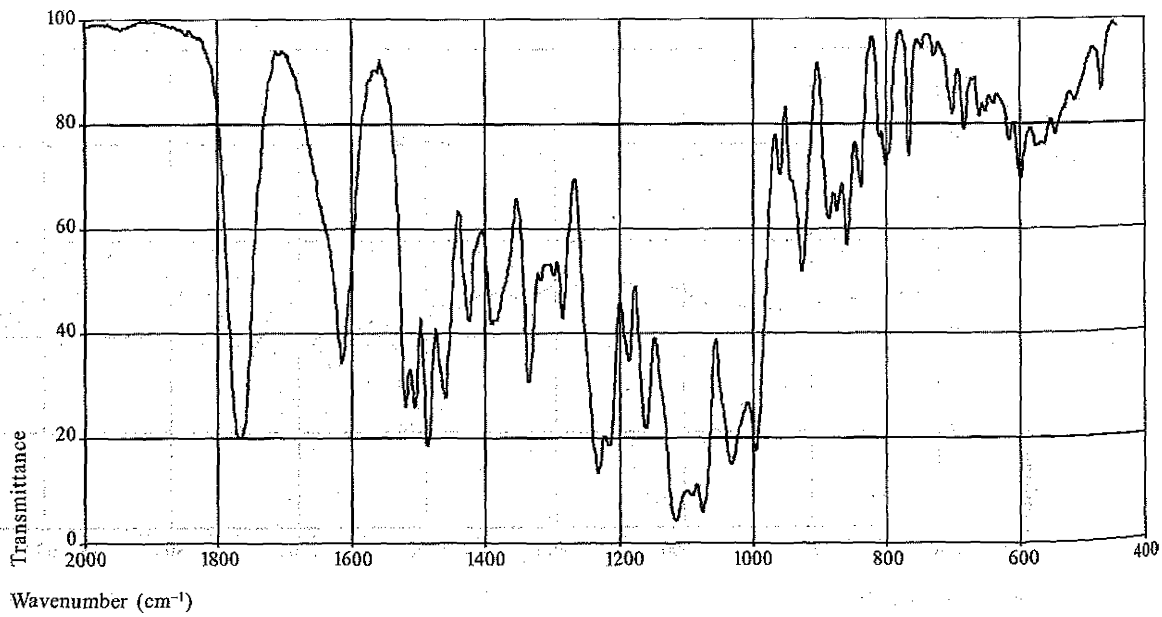
Etidronate Disodium *FTIR* Phase: KBr disc



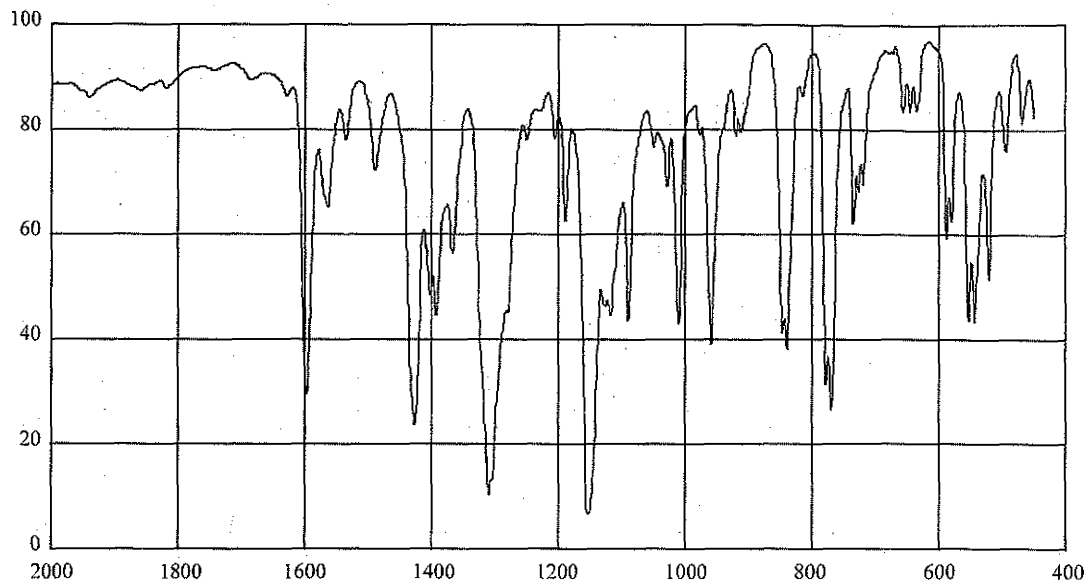
Etodolac *FTIR* Phase: KBr disc



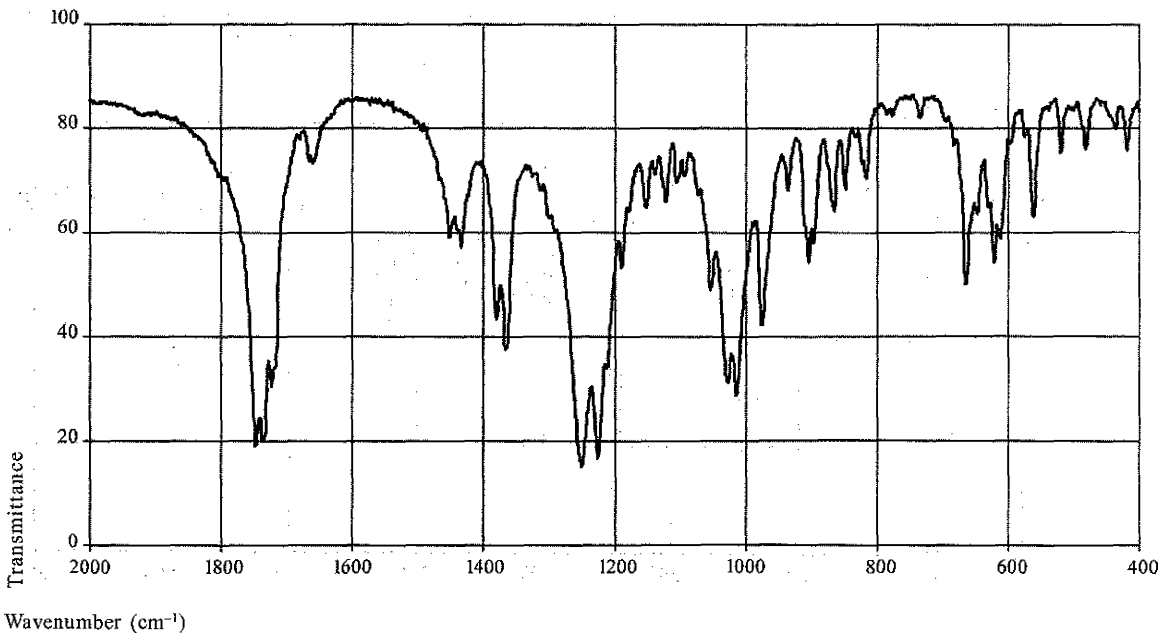
Etoposide *FTIR* Phase: KBr disc



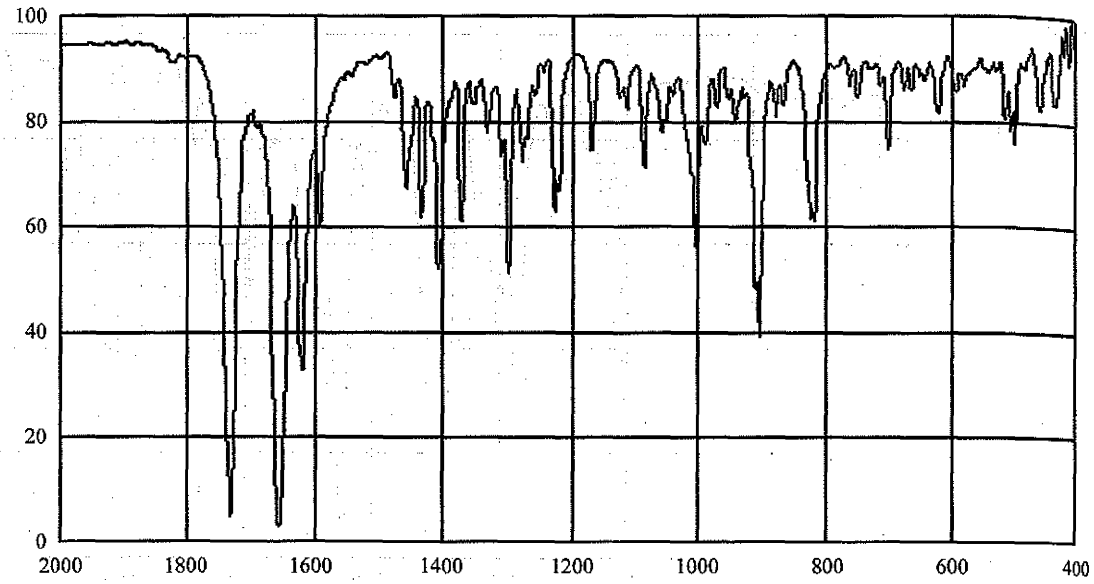
Etoricoxib *FTIR* Phase: KBr disc



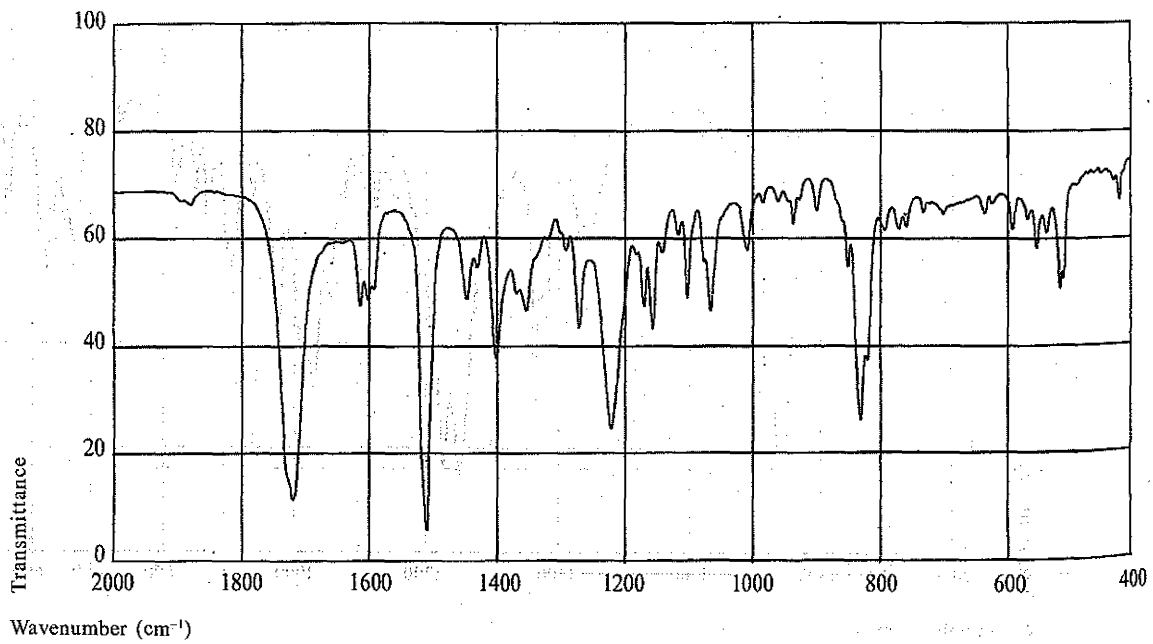
Ethynodiol Diacetate *Dispersive IR* Phase: KBr disc



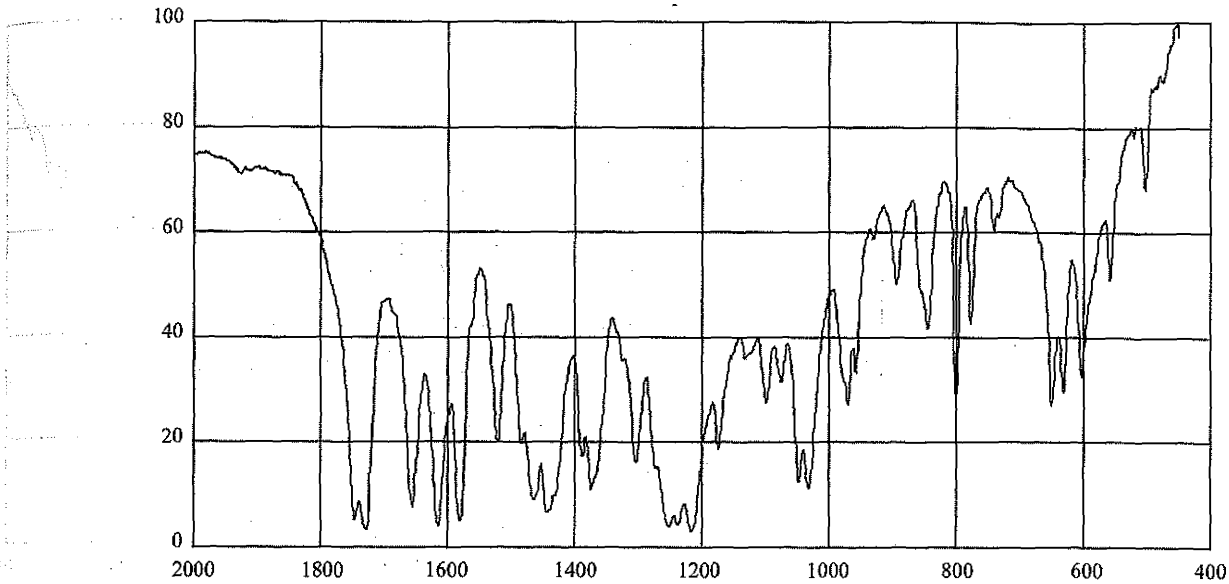
Exemestane *FTIR* Phase: KBr disc



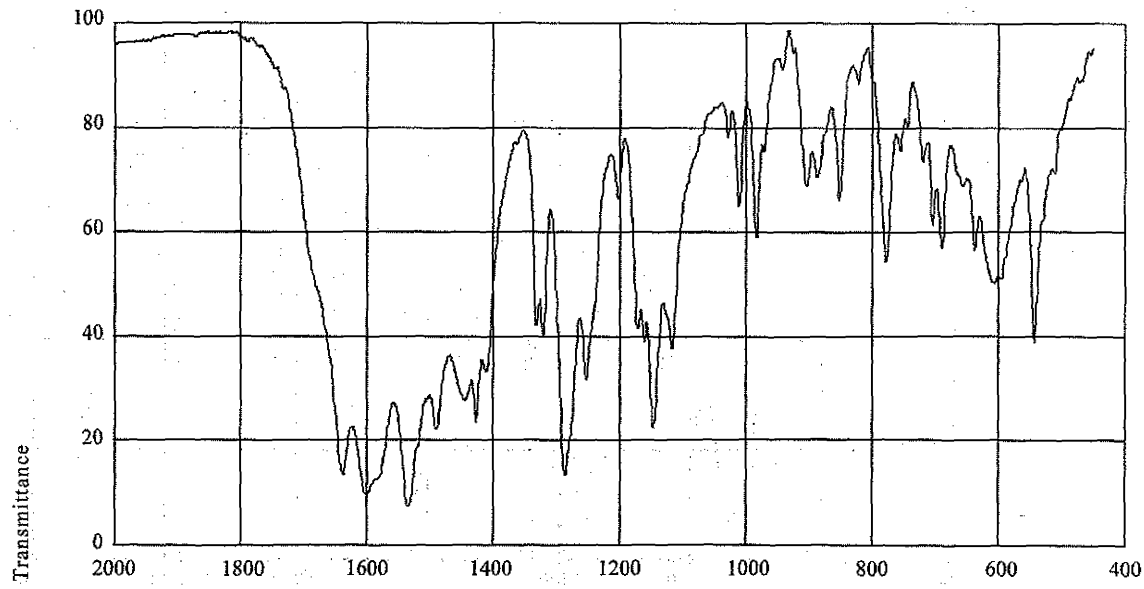
Ezetimibe *FTIR* Phase: KBr disc



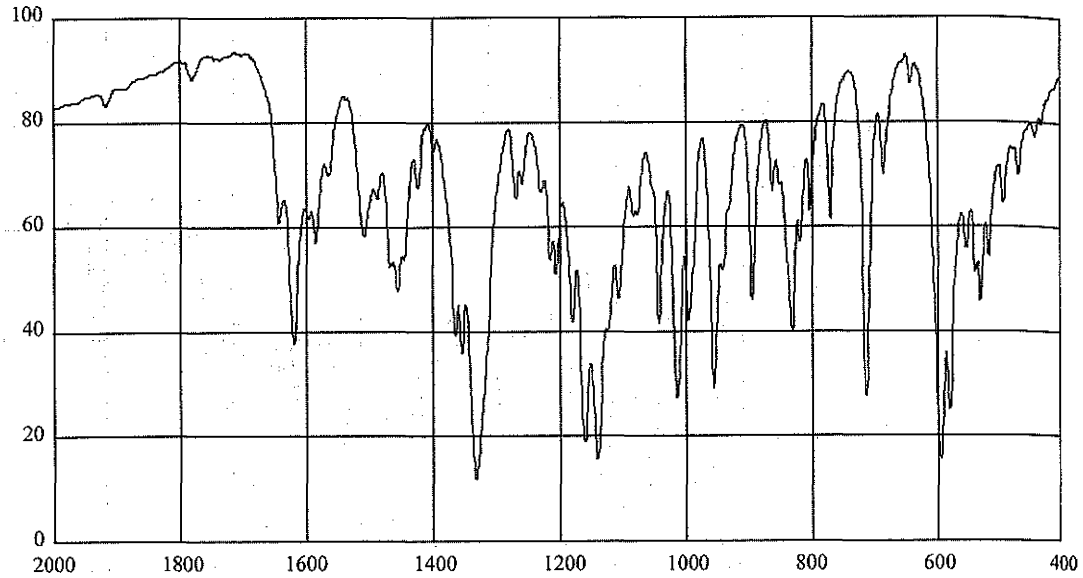
Famciclovir *FTIR* Phase: KBr disc



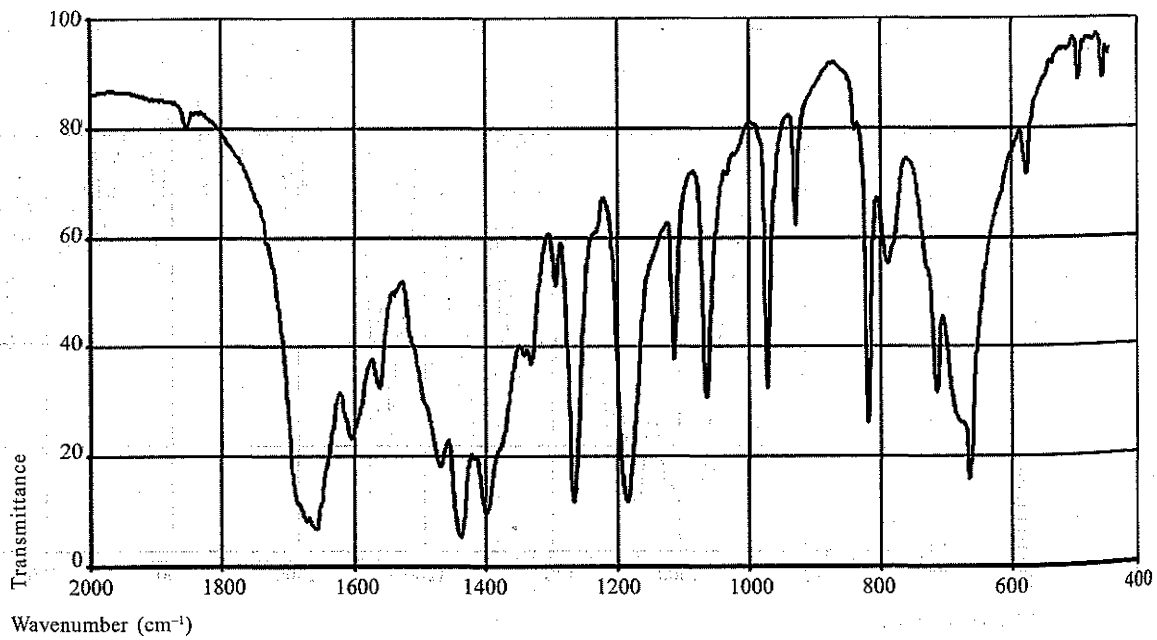
Famotidine *FTIR* Phase: KBr disc



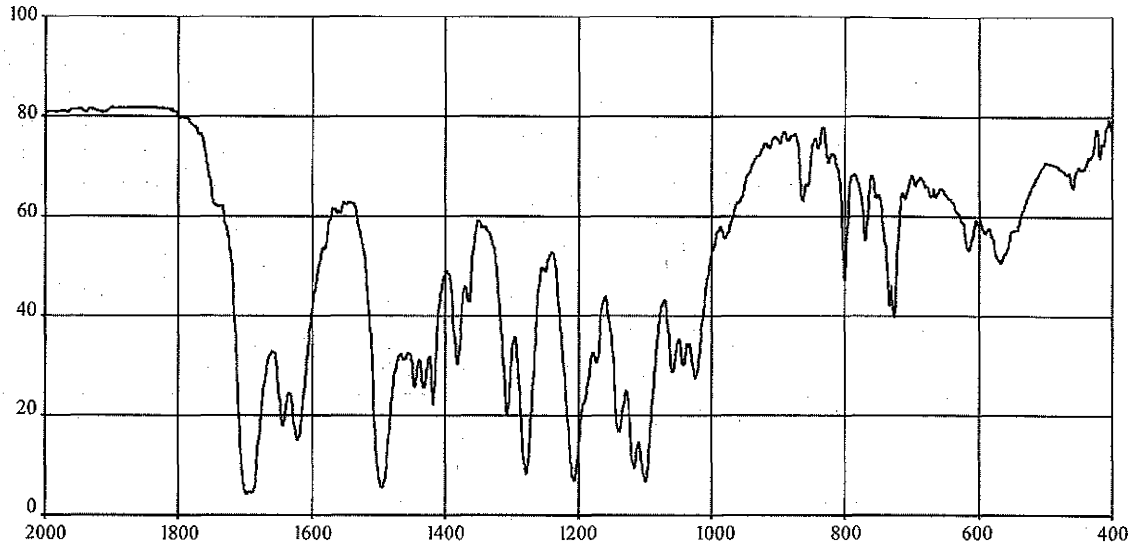
Fasudil Hydrochloride *FTIR* Phase: KBr disc



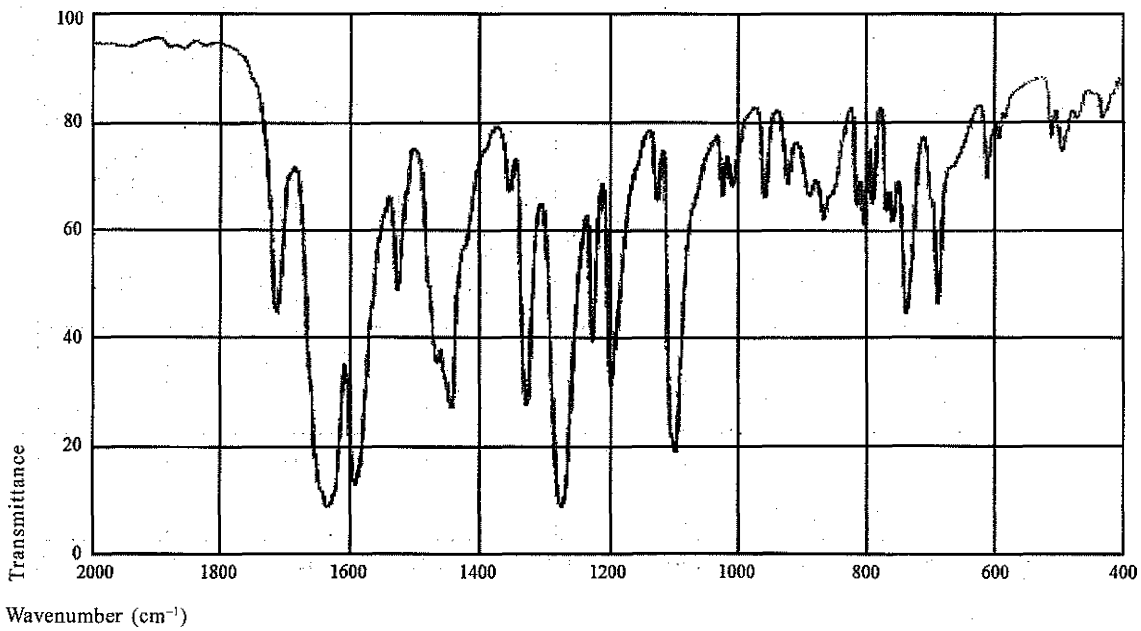
Favipiravir *FTIR* Phase: KBr disc



Felodipine *FTIR* Phase: KBr disc



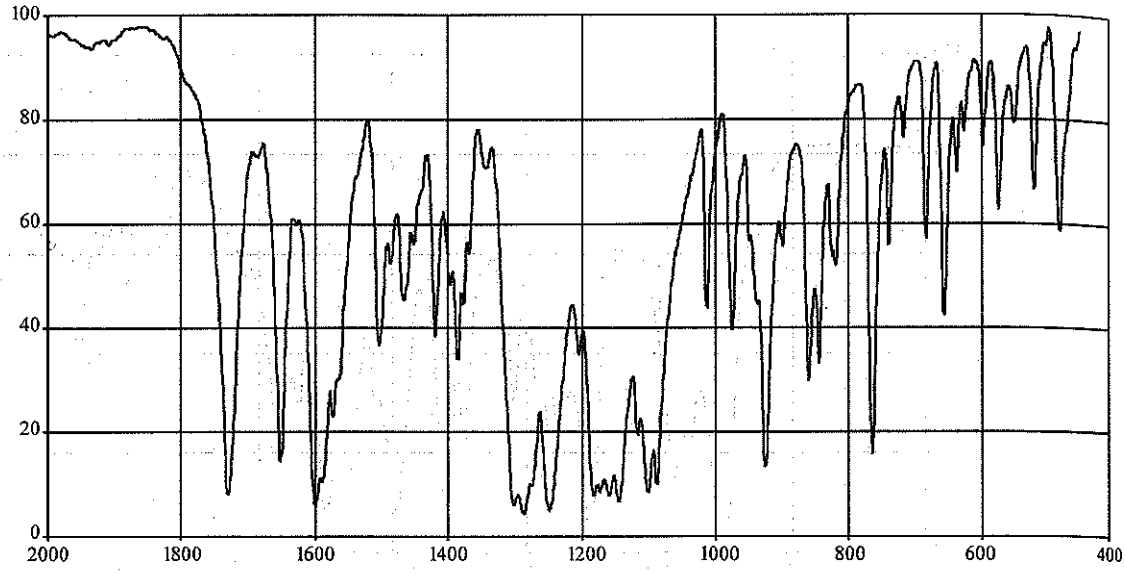
Fenbendazole *FTIR* Phase: KBr disc



Fenofibrate

FTIR

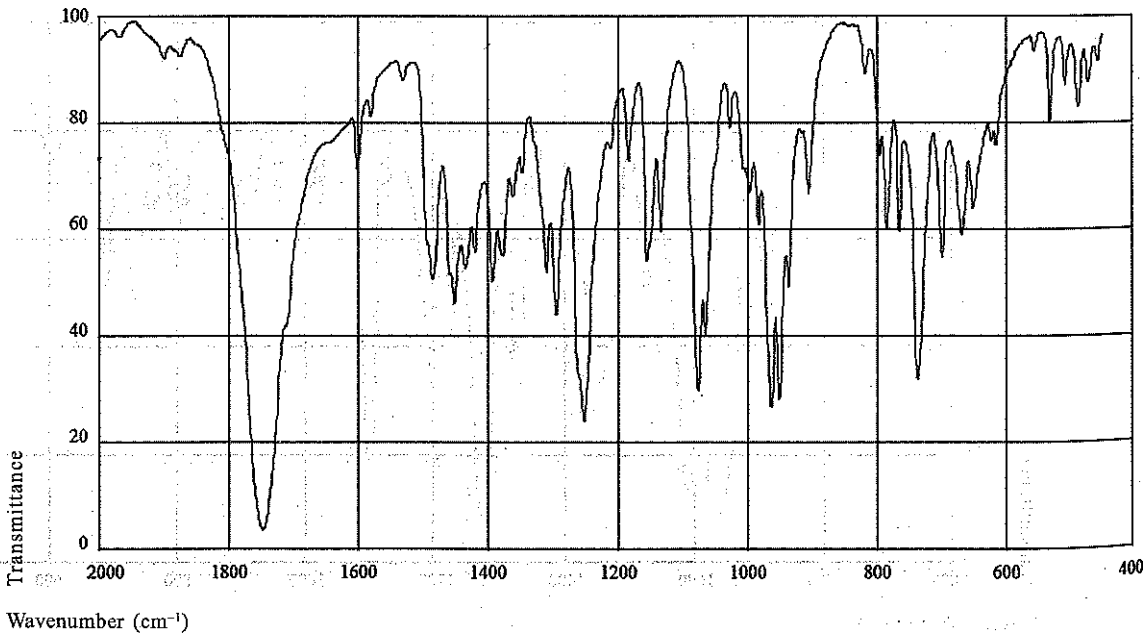
Phase: KBr disc



Fenspiride Hydrochloride

FTIR

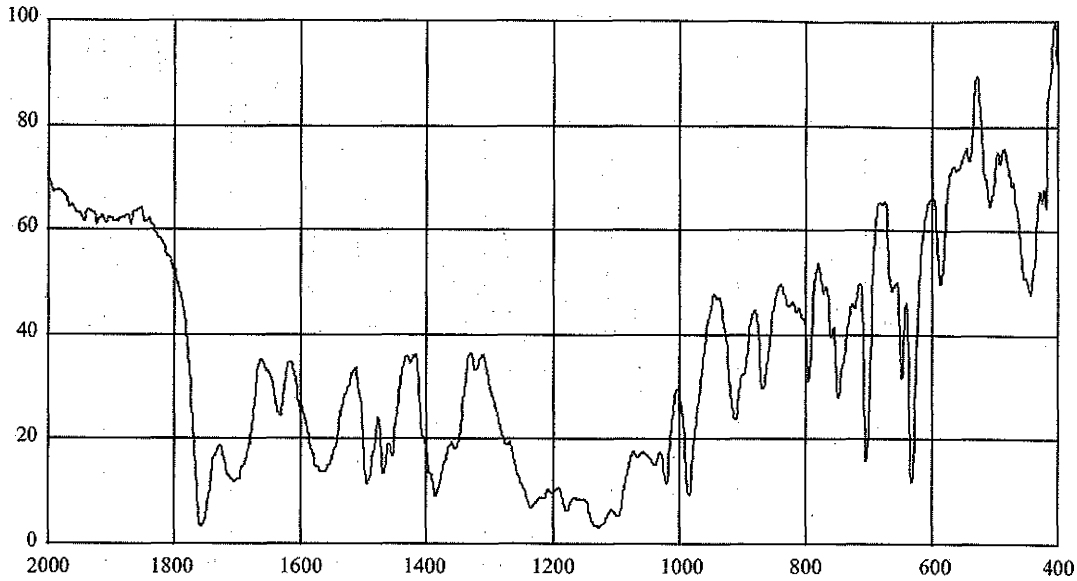
Phase: KBr disc



Fesoterodine Fumarate

FTIR

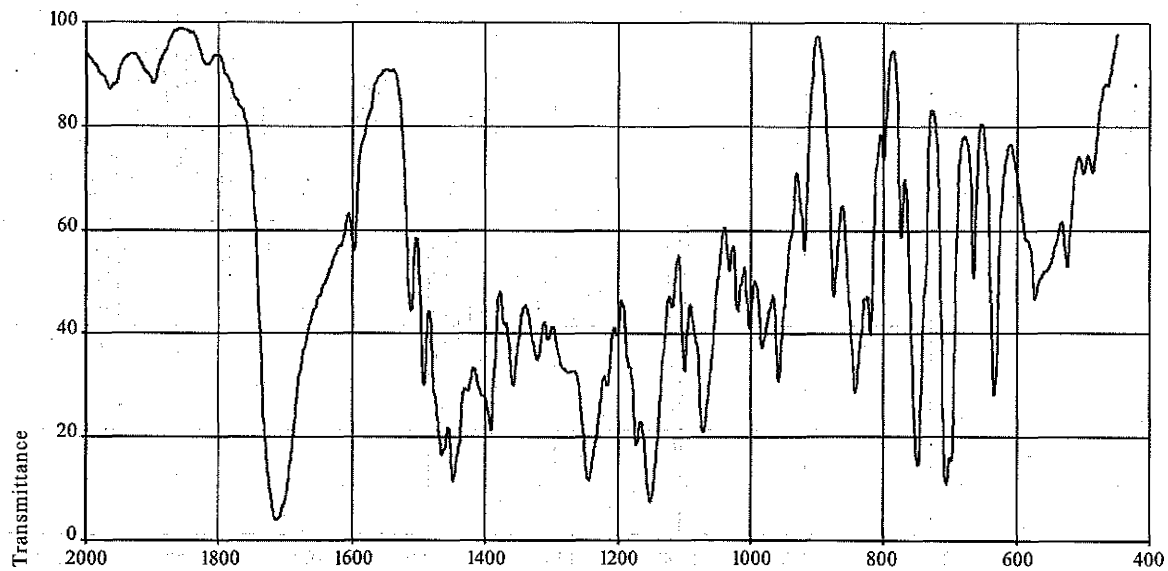
Phase: KBr disc



Fexofenadine Hydrochloride

FTIR

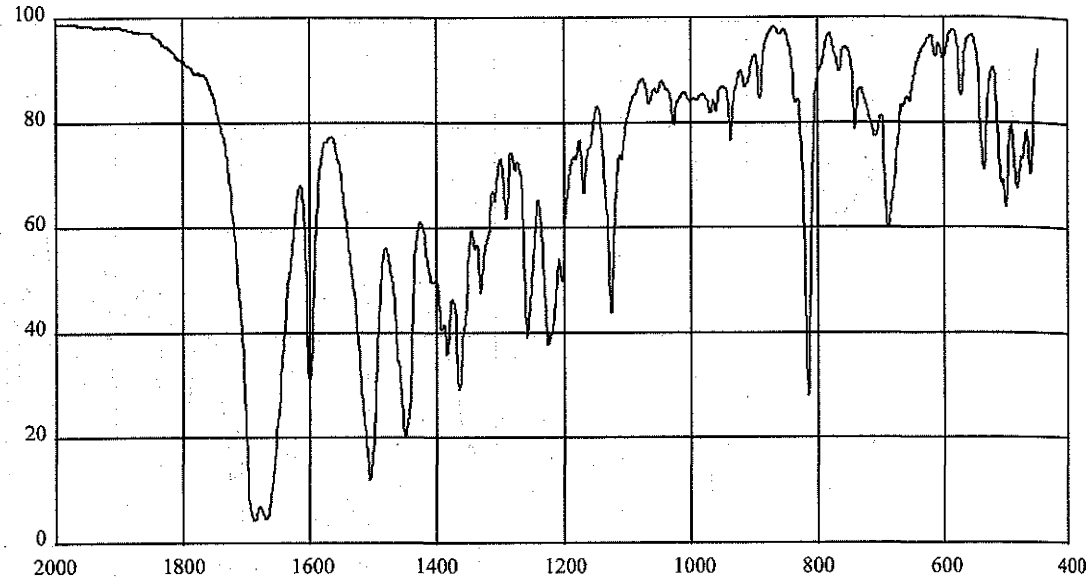
Phase: KBr disc



Finasteride

FTIR

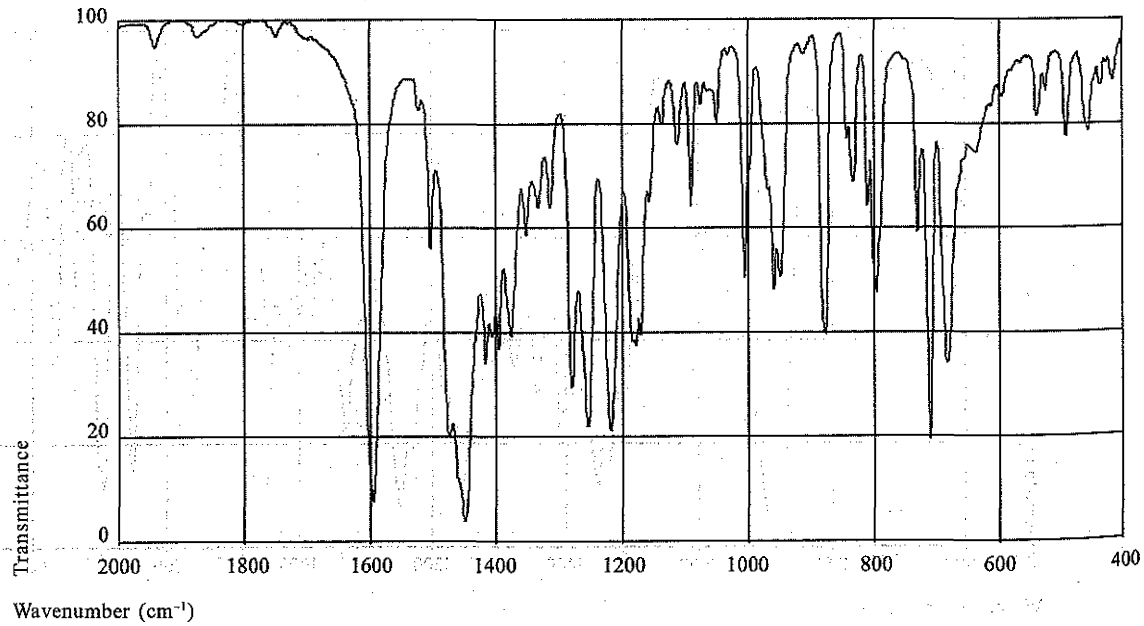
Phase: KBr disc



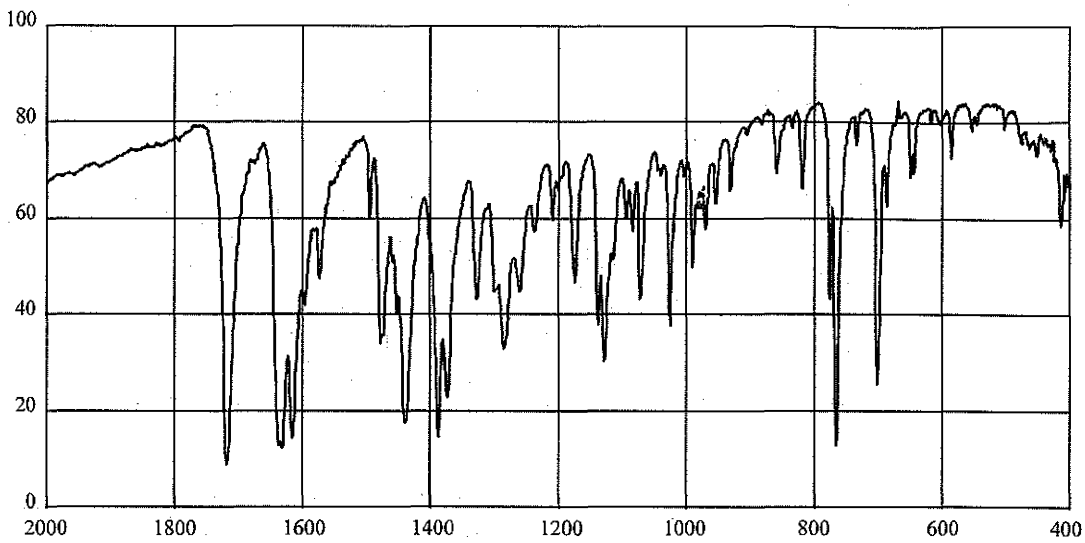
Fingolimod Hydrochloride

FTIR

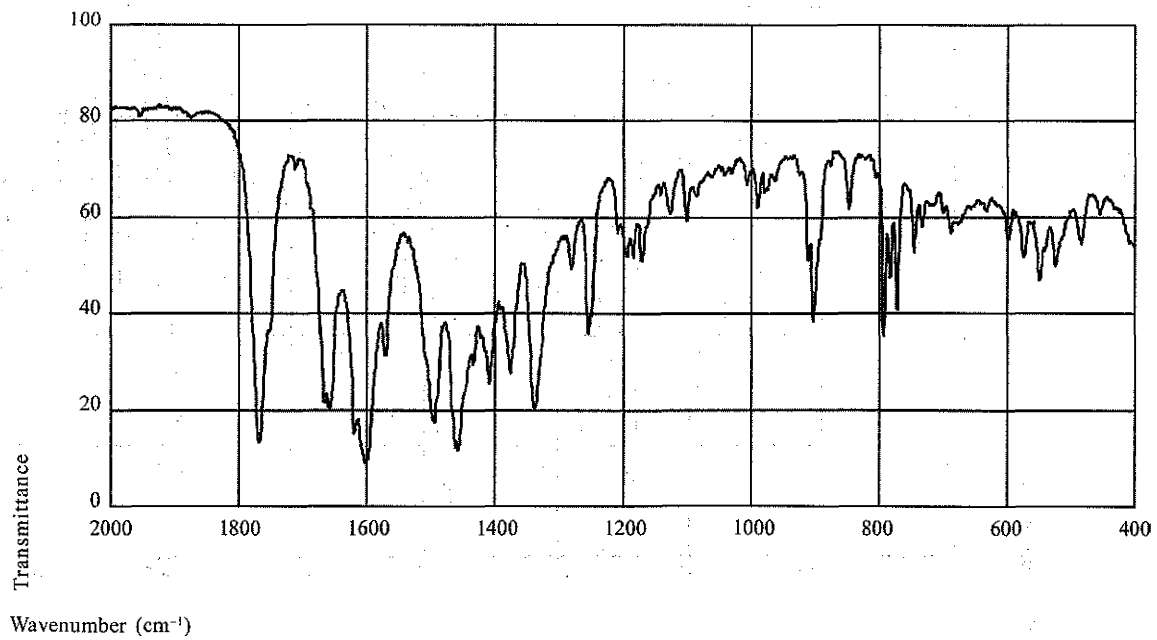
Phase: KBr disc



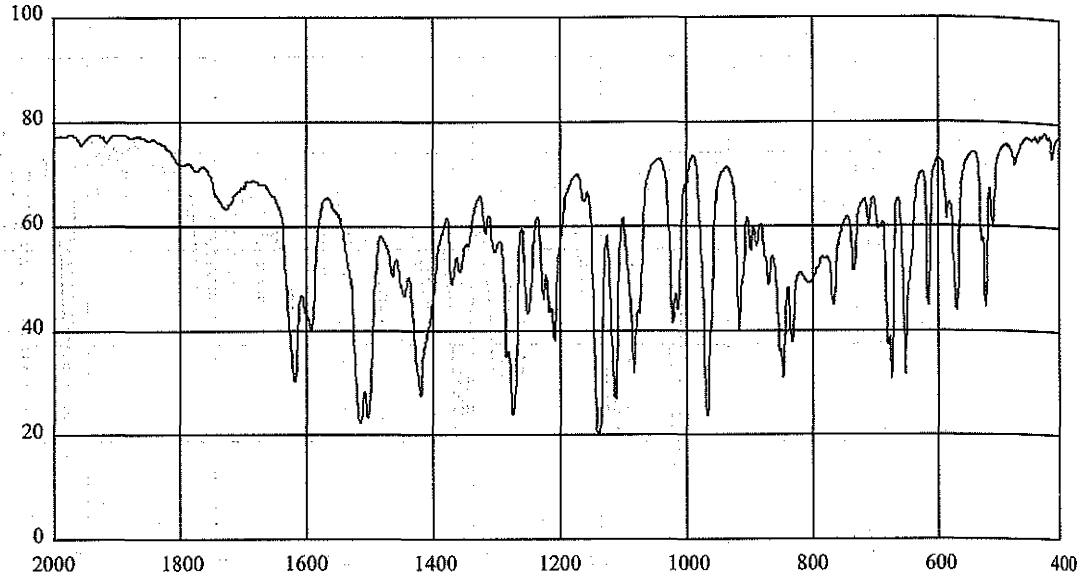
Flavoxate Hydrochloride *FTIR* Phase: KBr disc



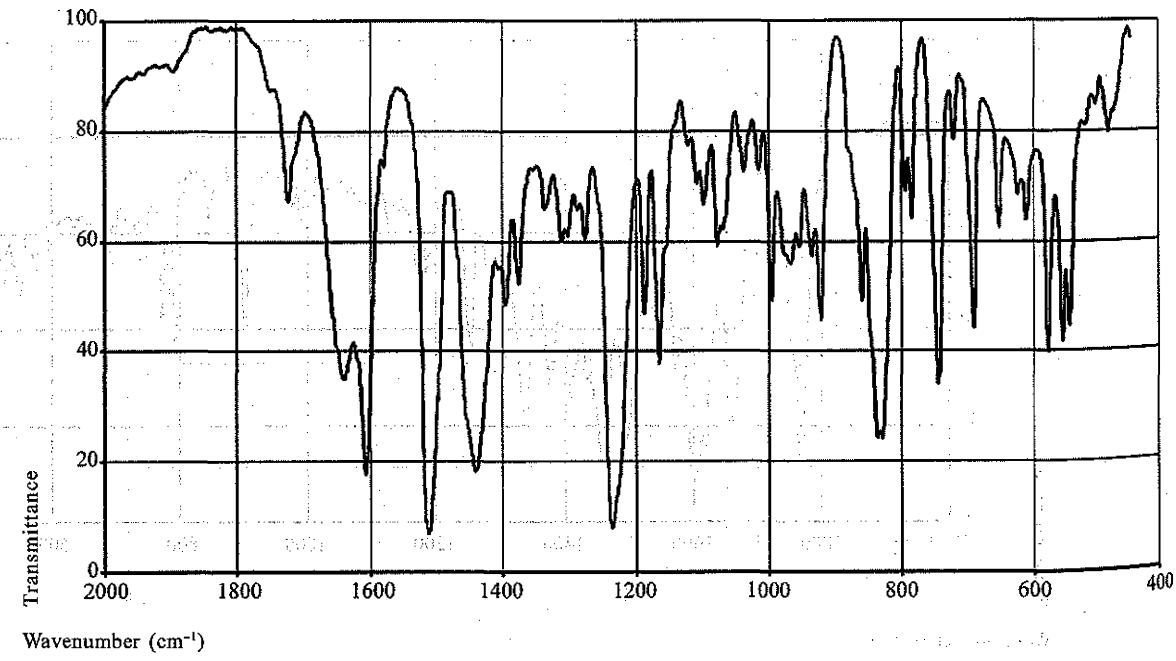
Flucloxacillin Sodium *FTIR* Phase: KBr disc



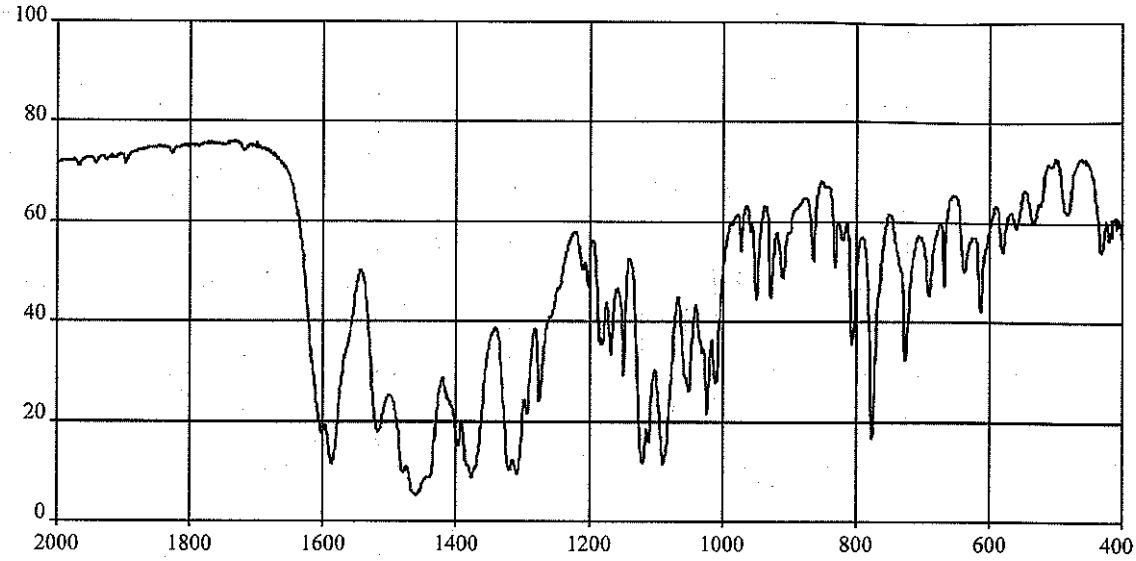
Fluconazole *FTIR* Phase: KBr disc



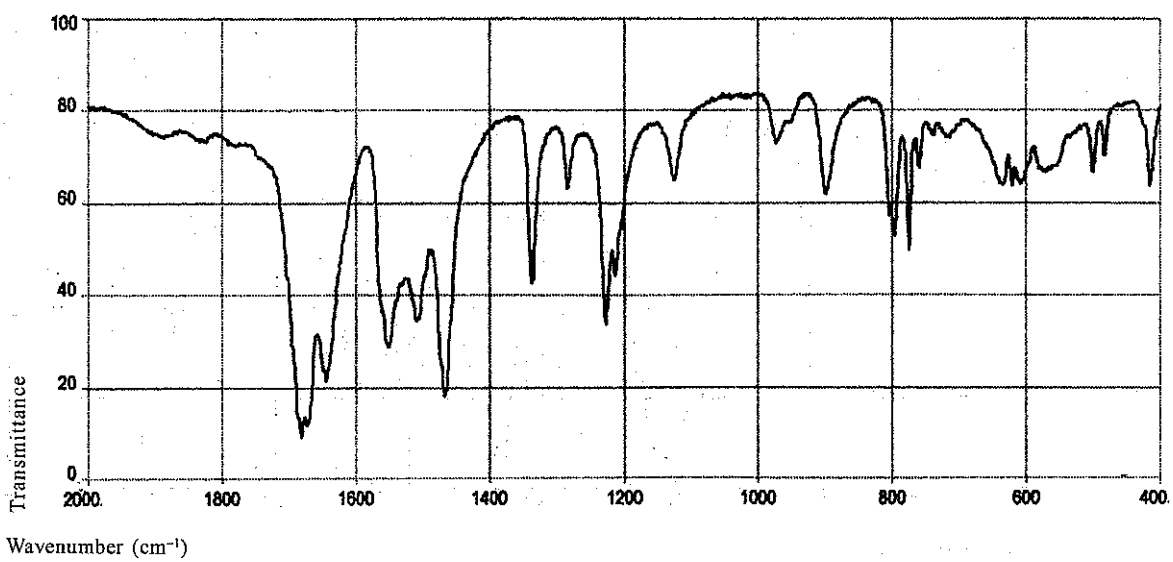
Flunarizine Dihydrochloride *FTIR* Phase: KBr disc



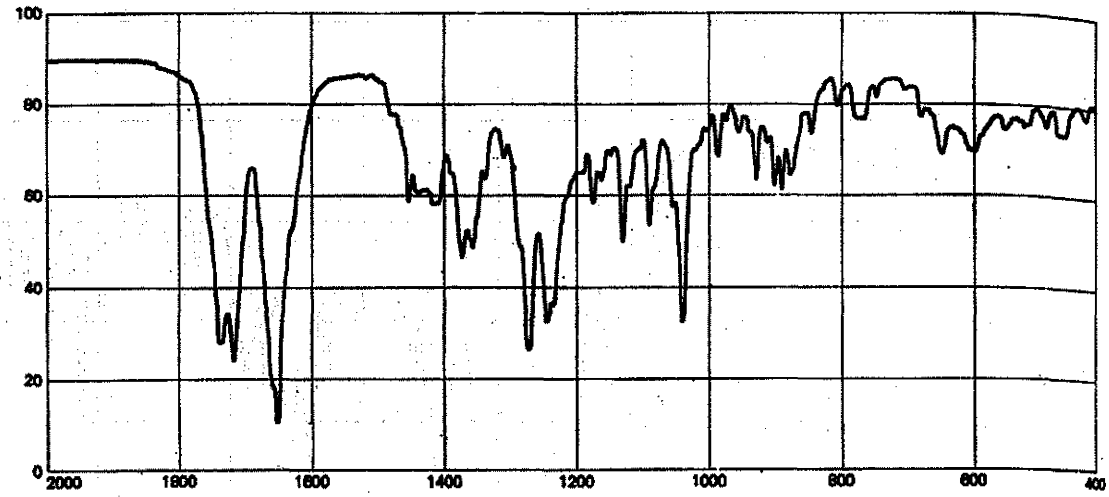
Flunixin Meglumine *FTIR* Phase: KBr disc



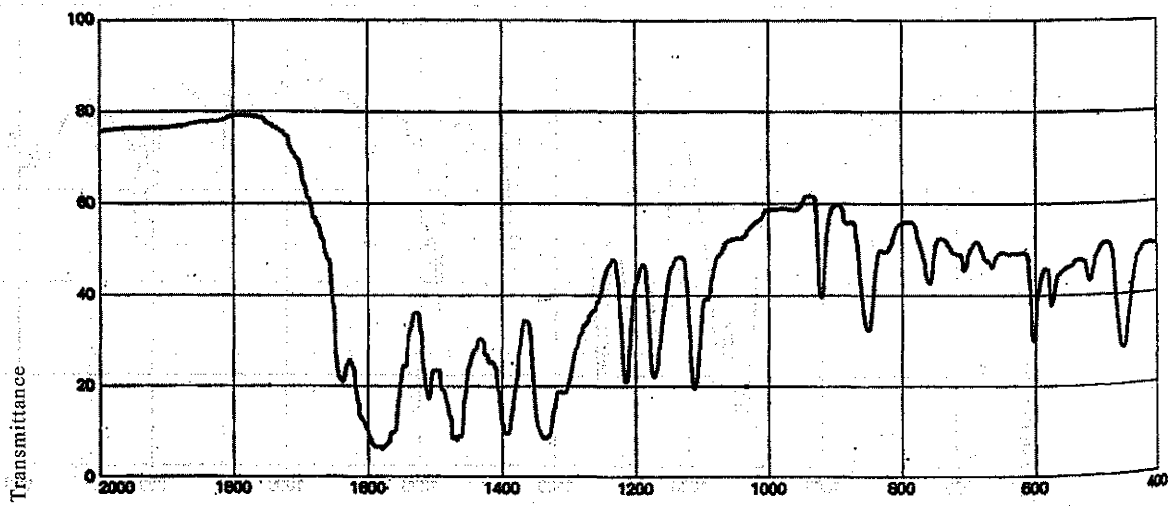
Flucytosine *FTIR* Phase: KBr disc



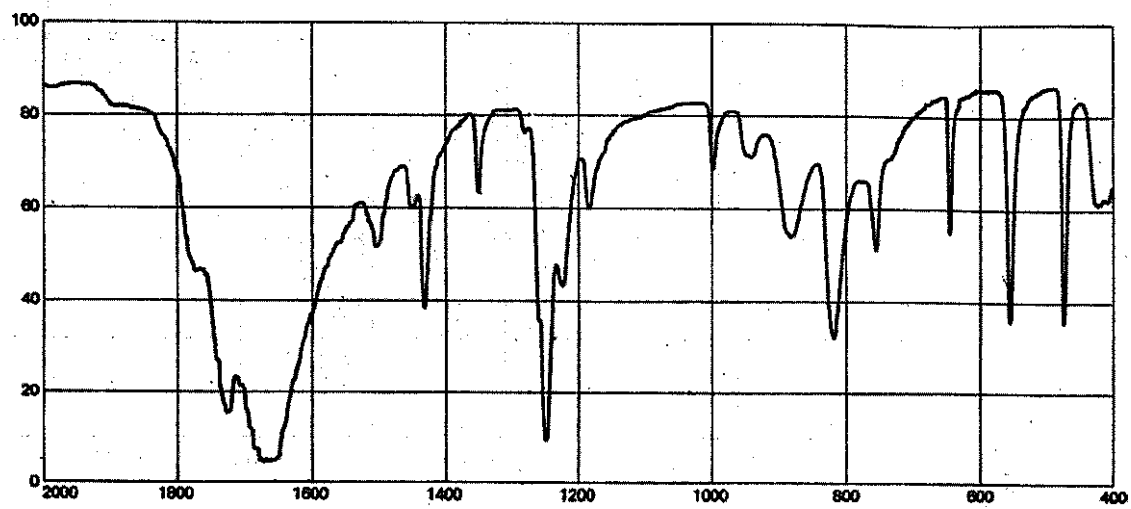
Fludrocortisone Acetate *Dispersive IR* Phase: KBr disc



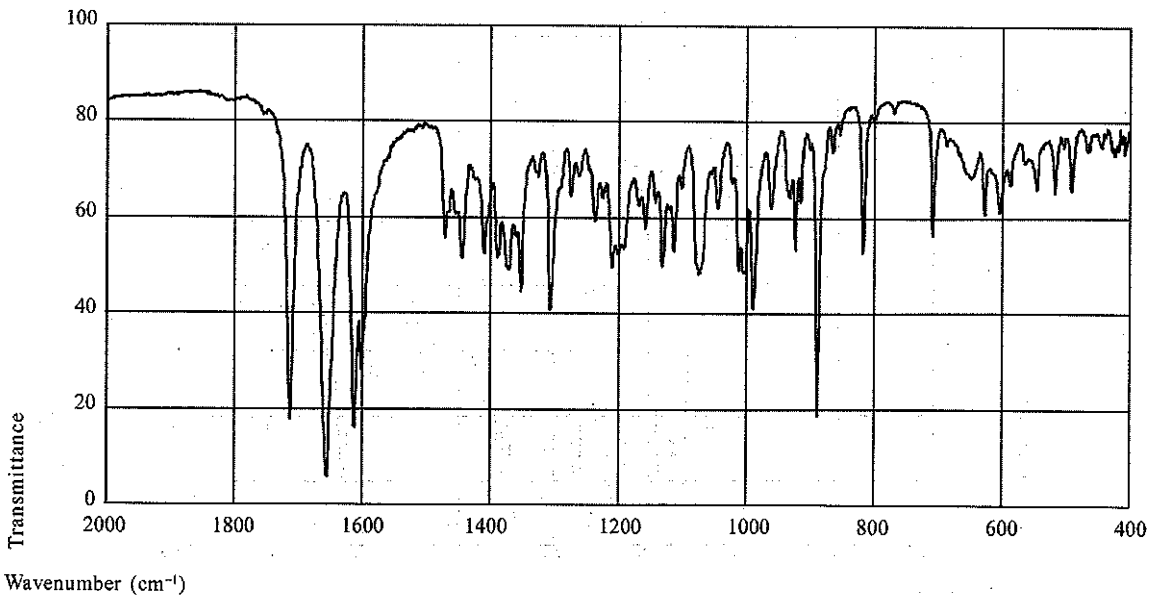
Fluorescein Sodium *Dispersive IR* Phase: KBr disc



Fluorouracil *Dispersive IR* Phase: KBr disc



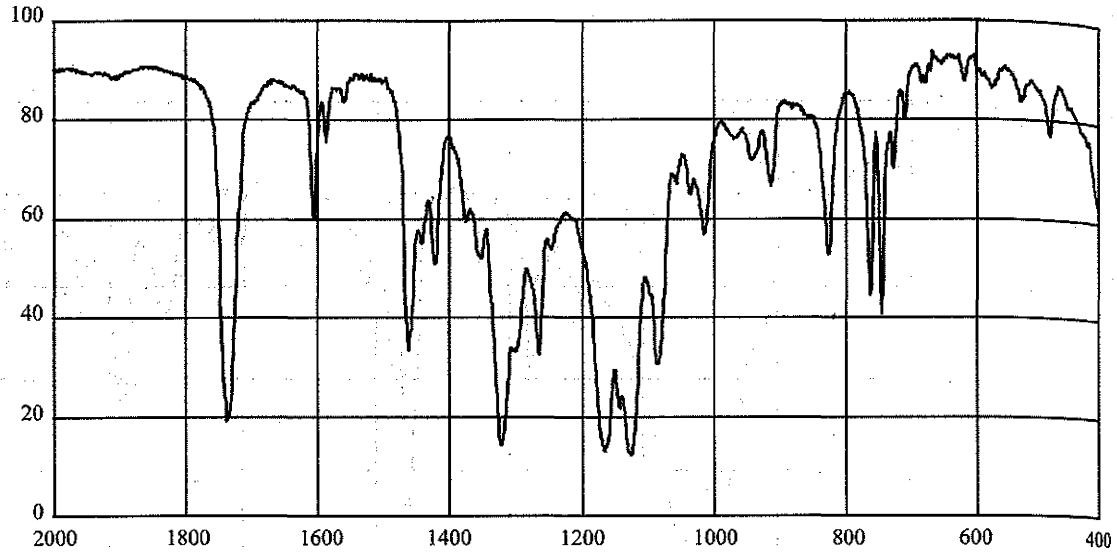
Fluoxetine Hydrochloride *FTIR* Phase: KBr disc



Flupentixol Decanoate

FTIR

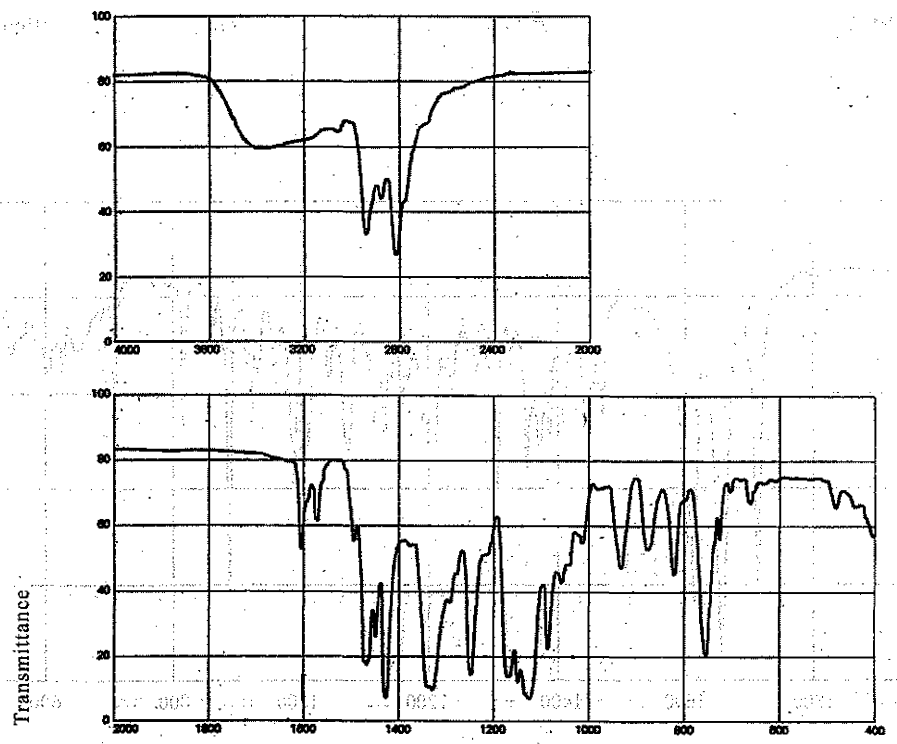
Phase: KBr disc



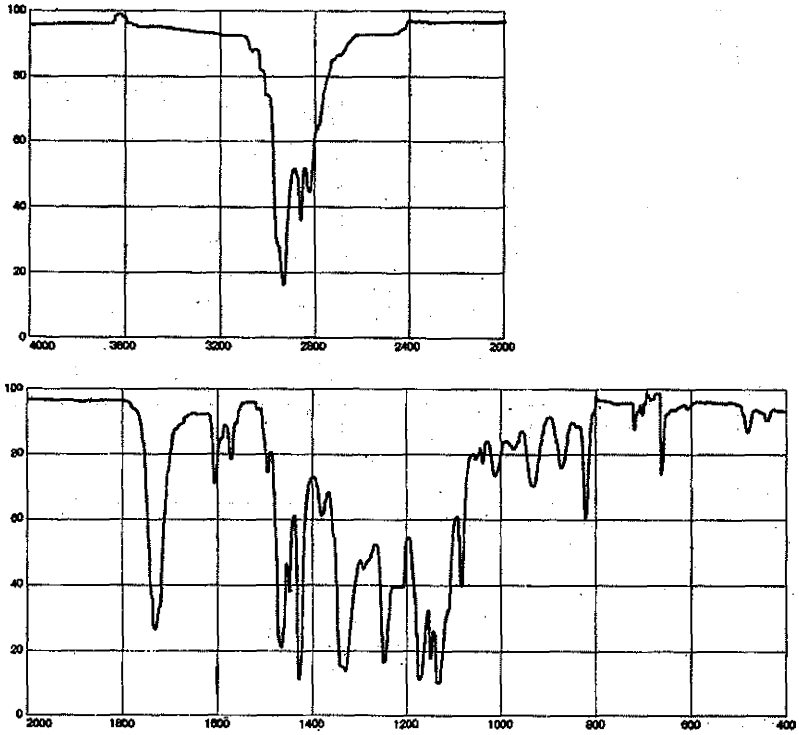
Fluphenazine

Dispersive IR

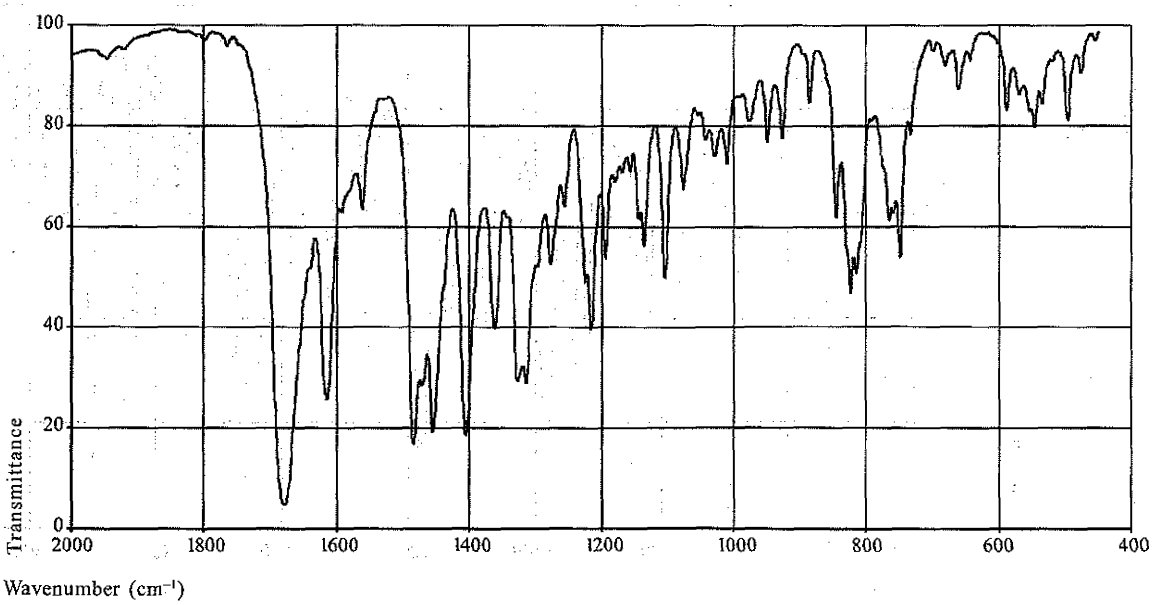
Phase: Thin film



Fluphenazine Decanoate *Dispersive IR* Phase: 8 per cent w/v solution in $CHCl_3$, Thickness 0.1 mm



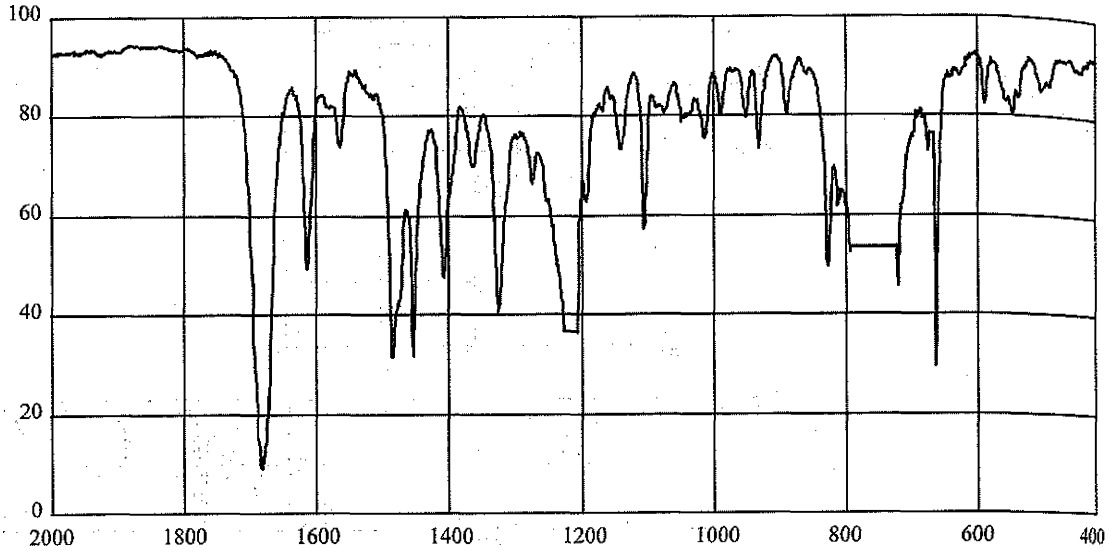
Flurazepam Hydrochloride *FTIR* Phase: KBr disc



Flurazepam Monohydrochloride

FTIR

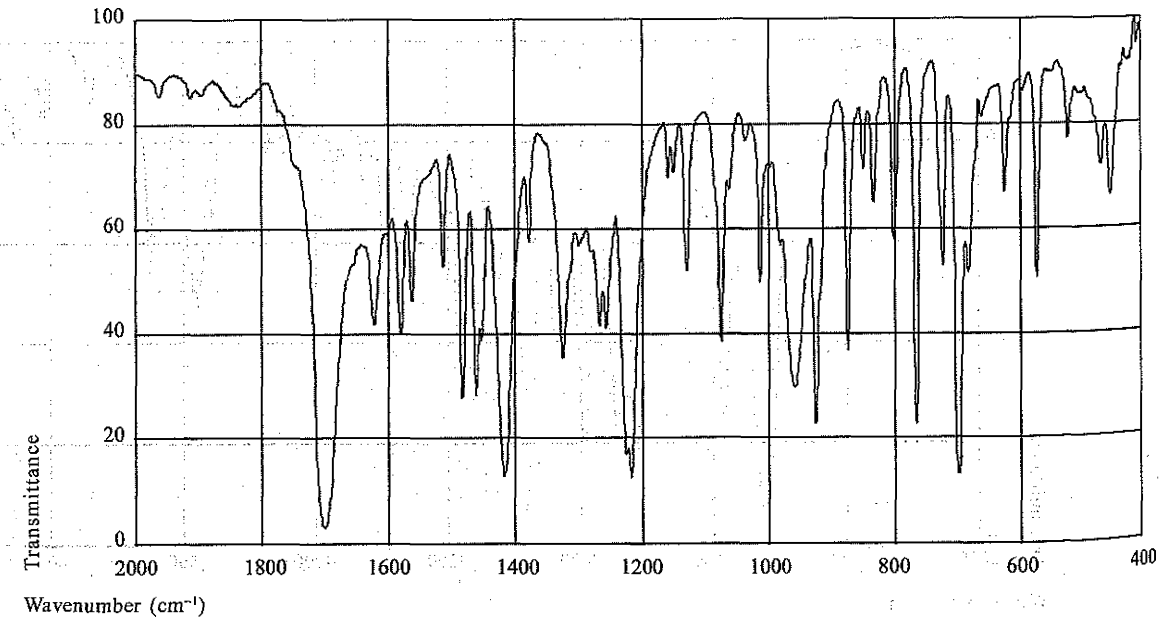
Phase: KBr disc



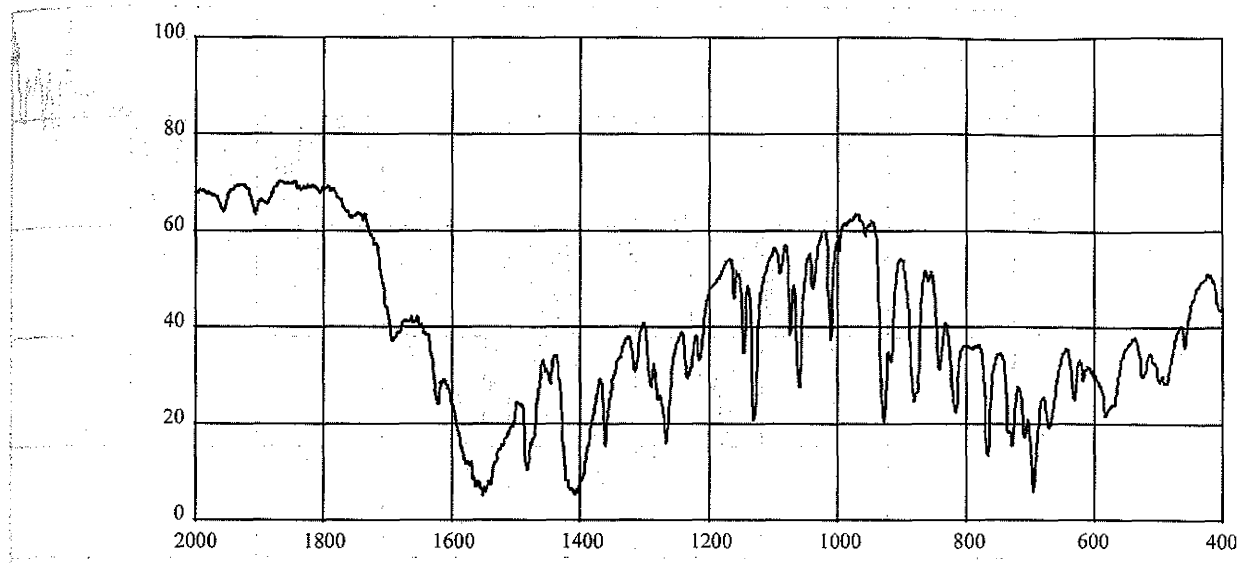
Flurbiprofen

FTIR

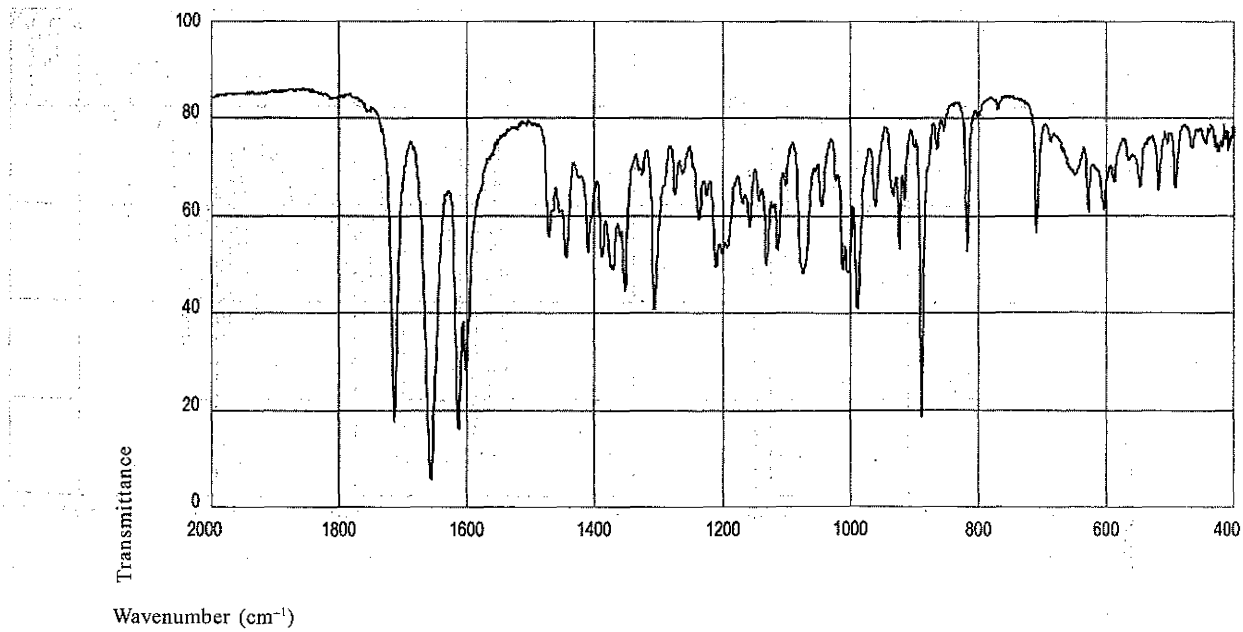
Phase: KBr disc



Flurbiprofen Sodium *FTIR* Phase: KBr disc



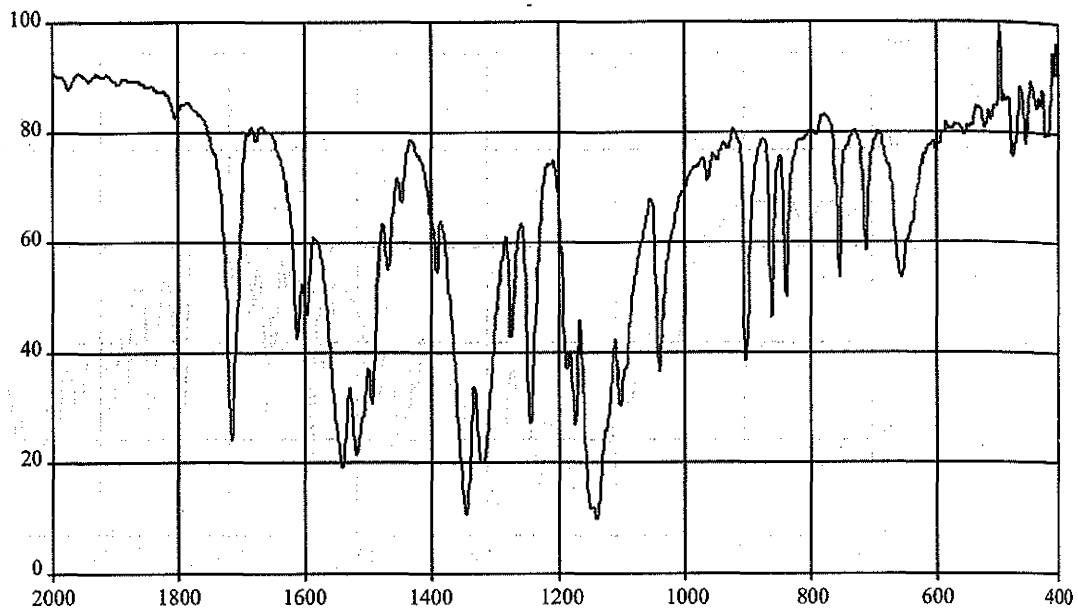
Fluorometholone *FTIR* Phase: KBr disc



Flutamide

FTIR

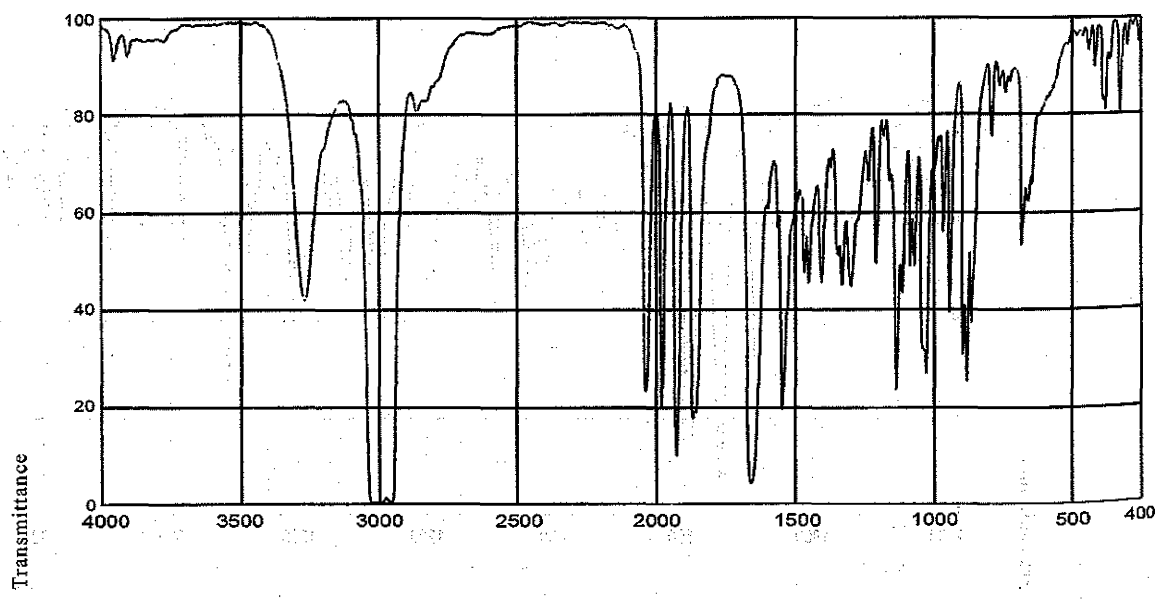
Phase: KBr disc



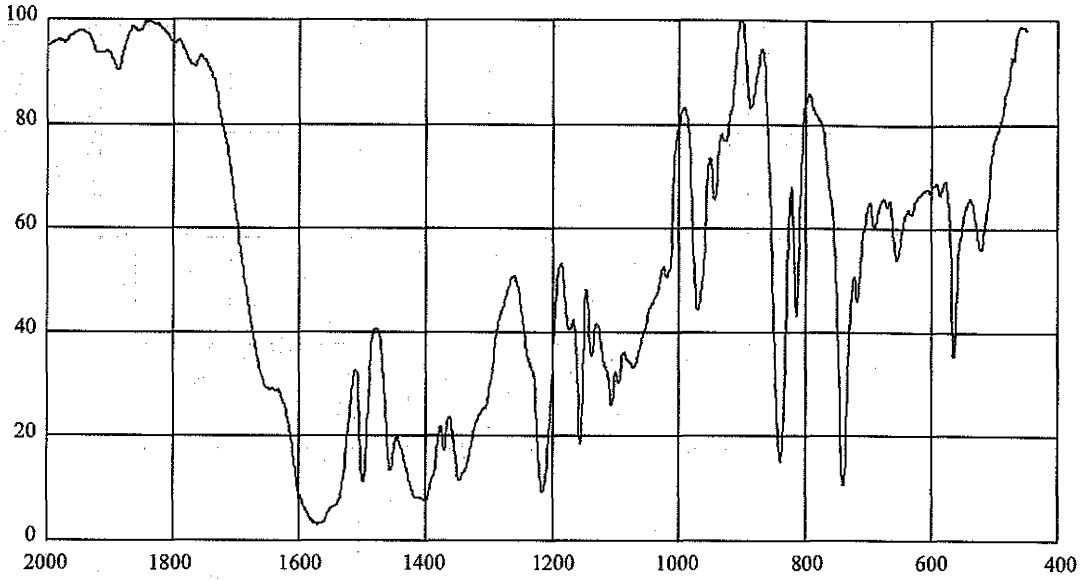
Fluticasone Propionate

FTIR

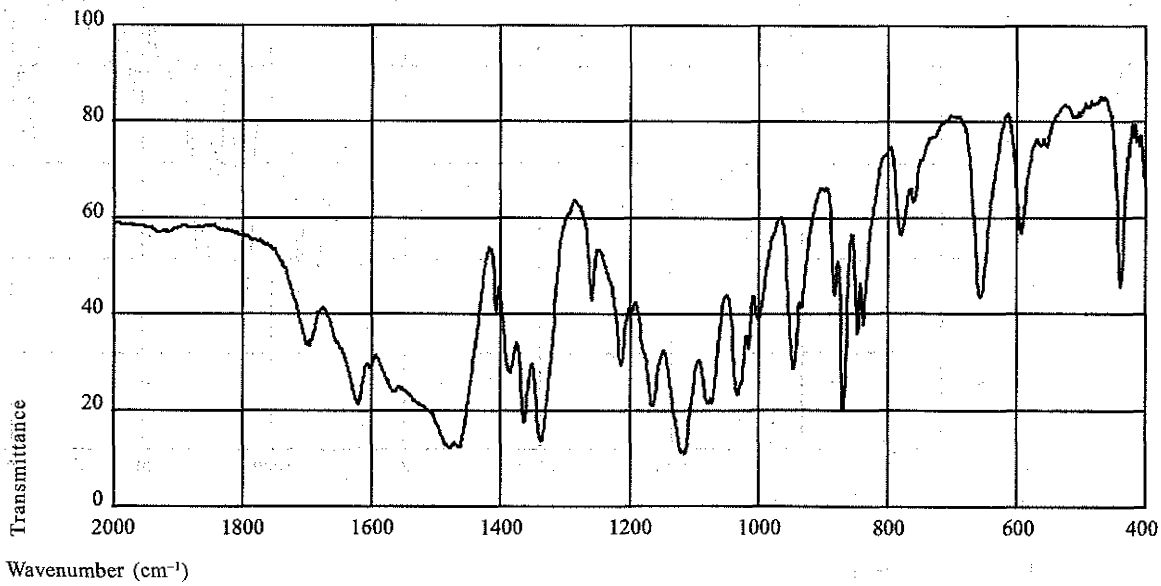
Phase: Liquid paraffin mull



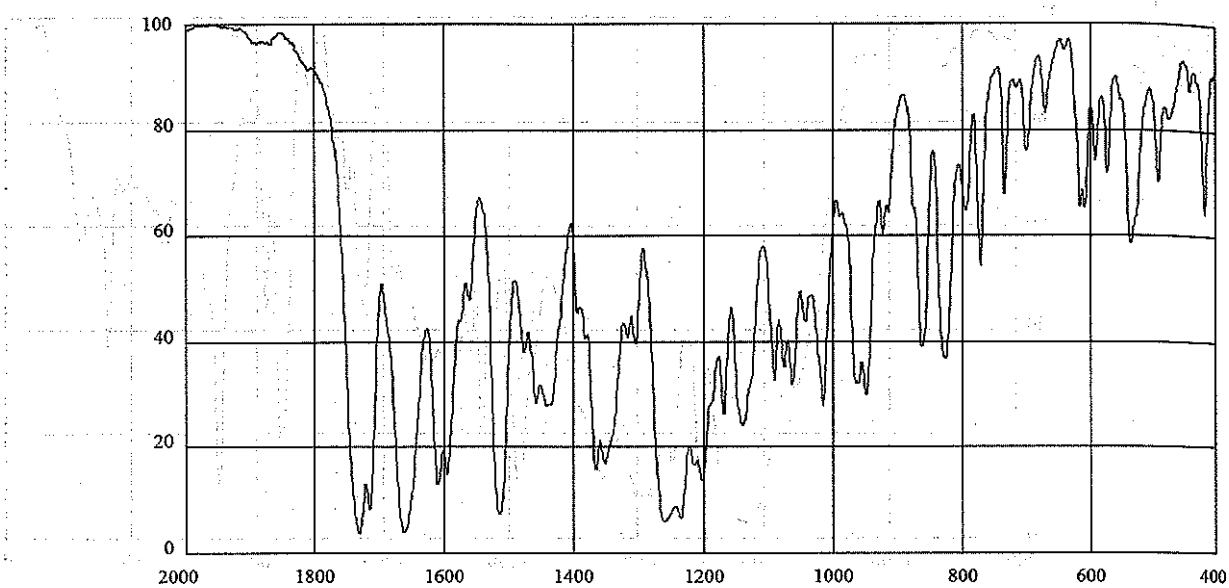
Fluvastatin Sodium *FTIR* Phase: KBr disc



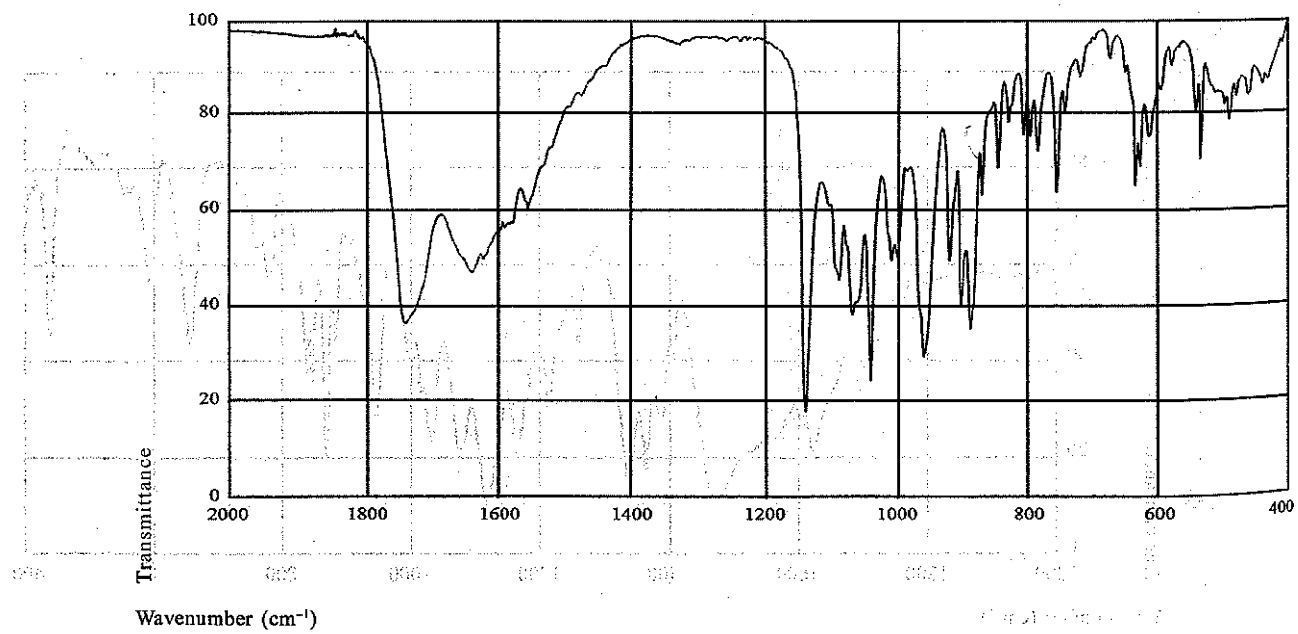
Fluvoxamine Maleate *FTIR* Phase: KBr disc



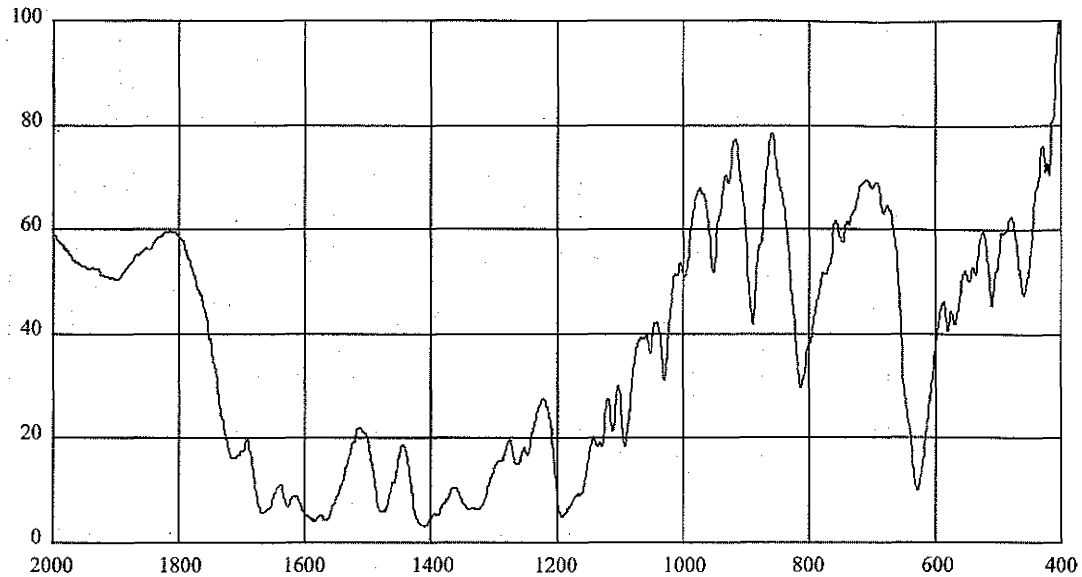
Fomepizole *FTIR* Phase: KBr disc



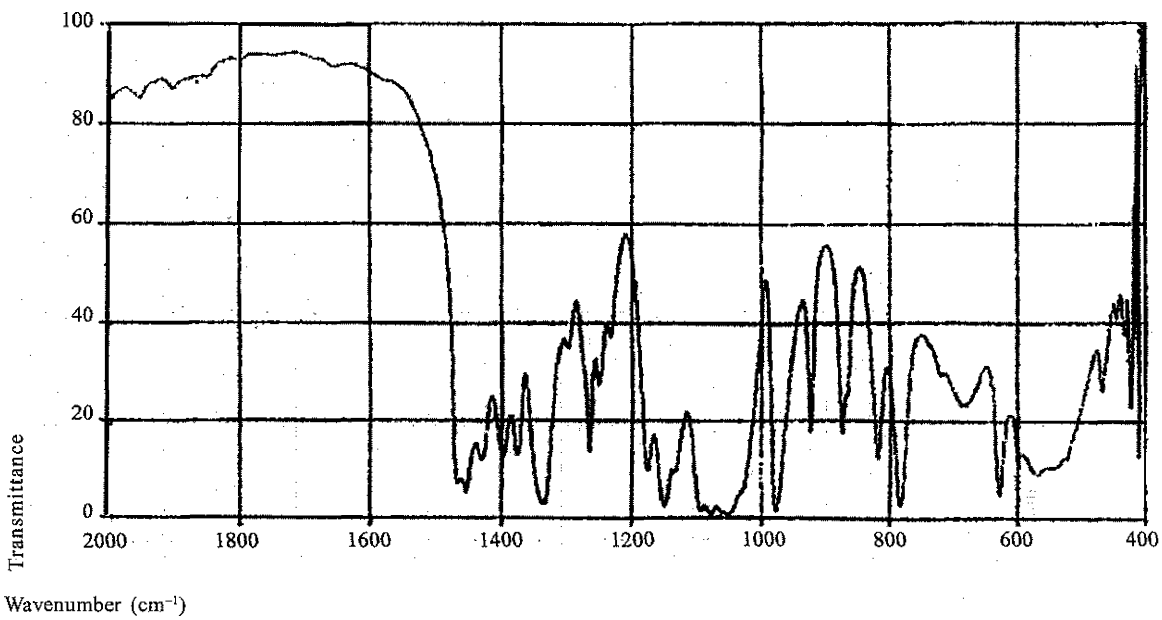
Formoterol Fumarate *FTIR* Phase: KBr disc



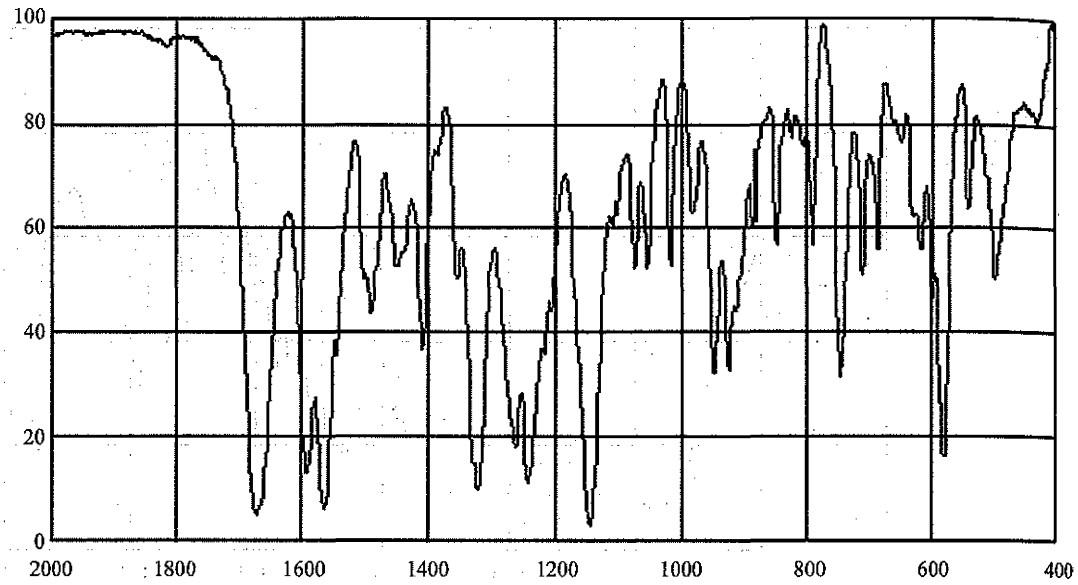
Frovatriptan Succinate *FTIR* Phase: KBr disc



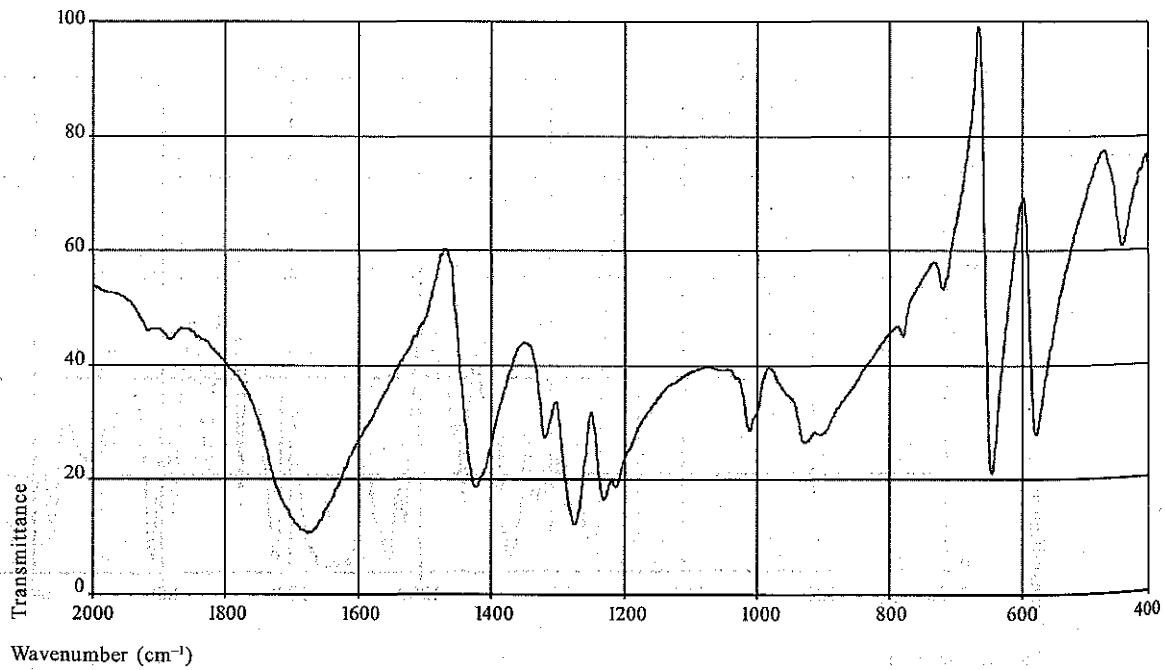
Fructose *FTIR* Phase: Liquid paraffin mull



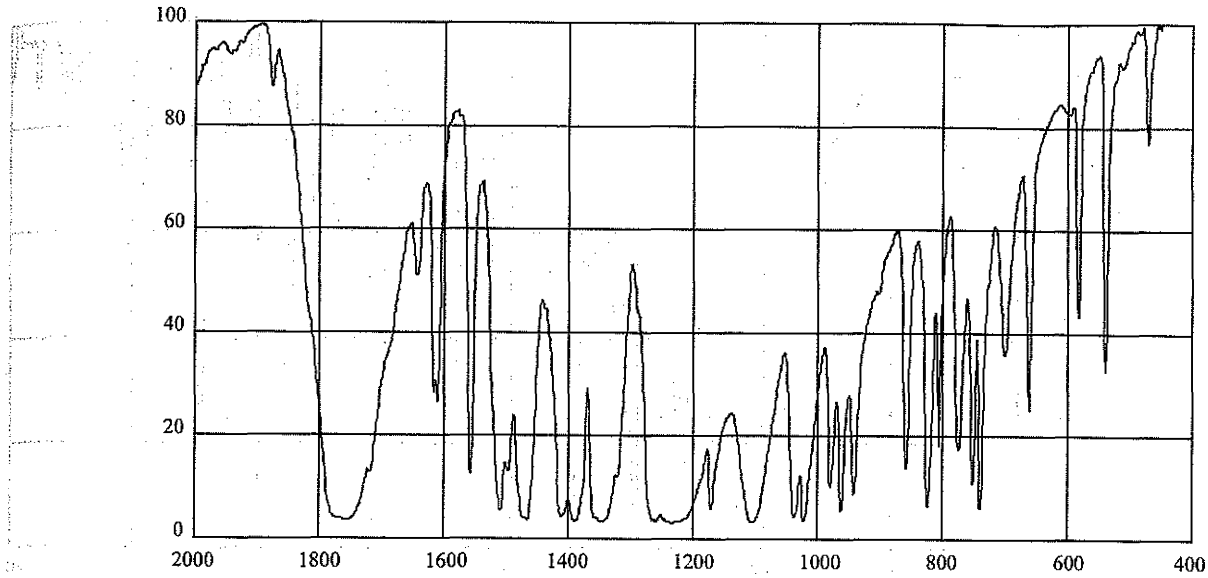
Frusemide *FTIR* Phase: KBr disc



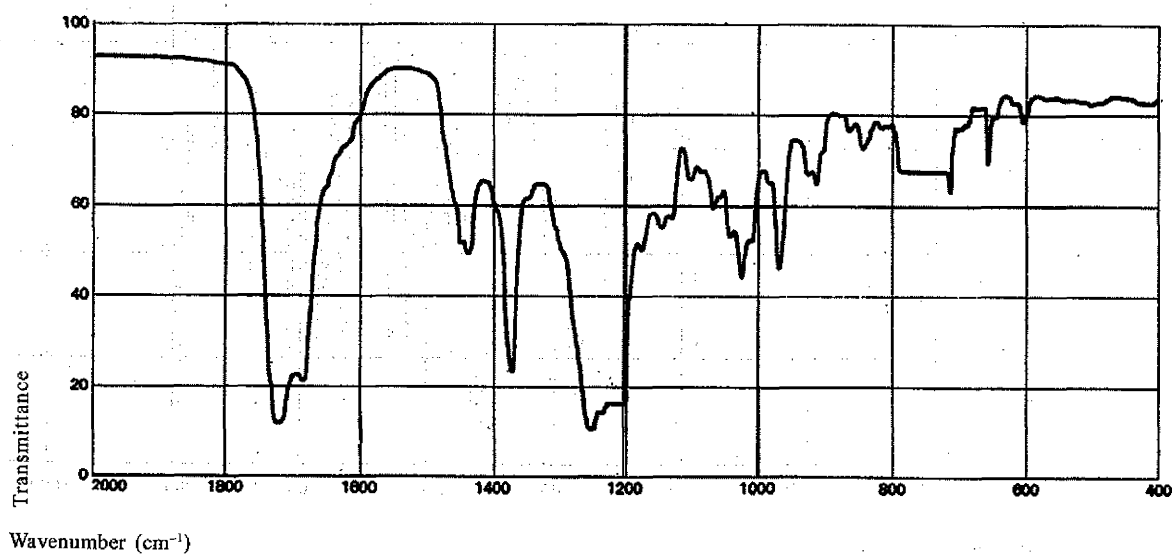
Fumaric Acid *FTIR* Phase: KBr disc



Furazolidone *FTIR* Phase: KBr disc



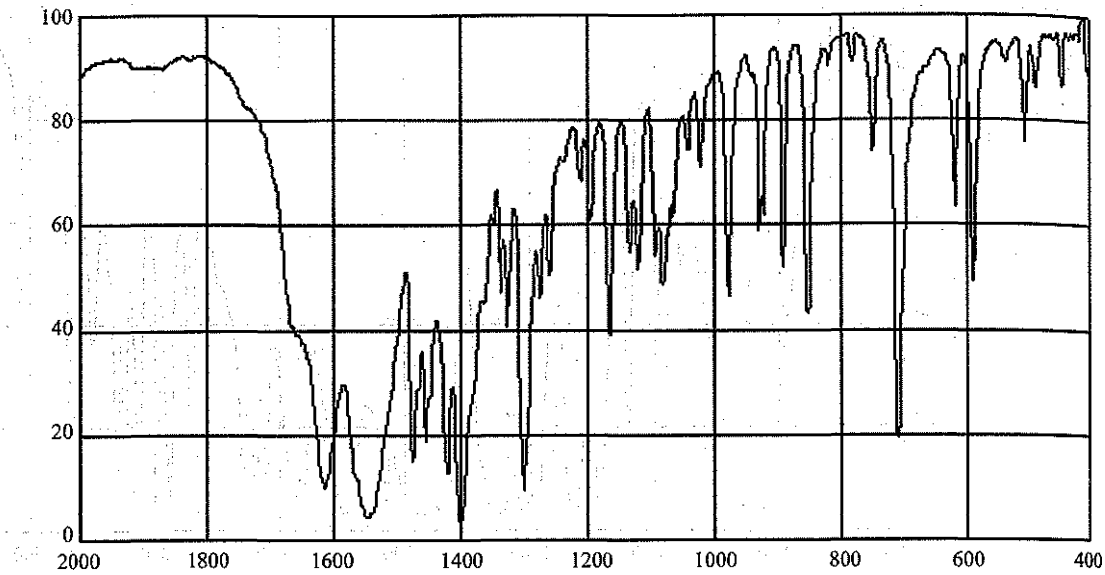
Fusidic Acid *Dispersive IR* Phase: 10 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



Gabapentin

FTIR

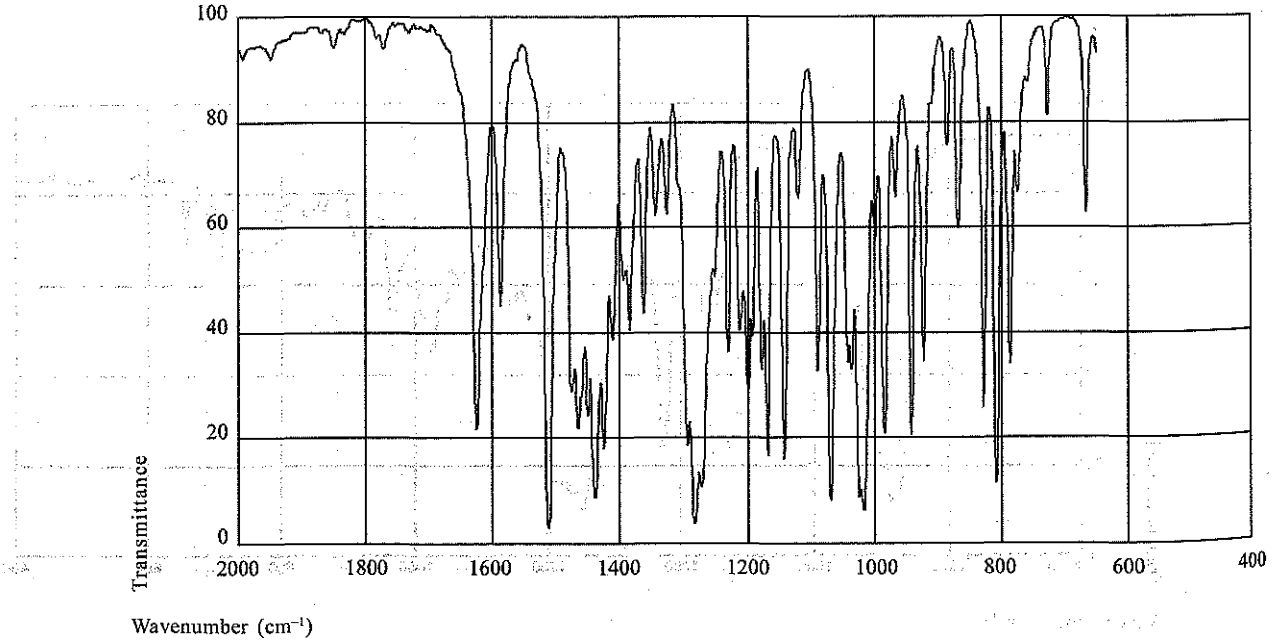
Phase: KBr disc



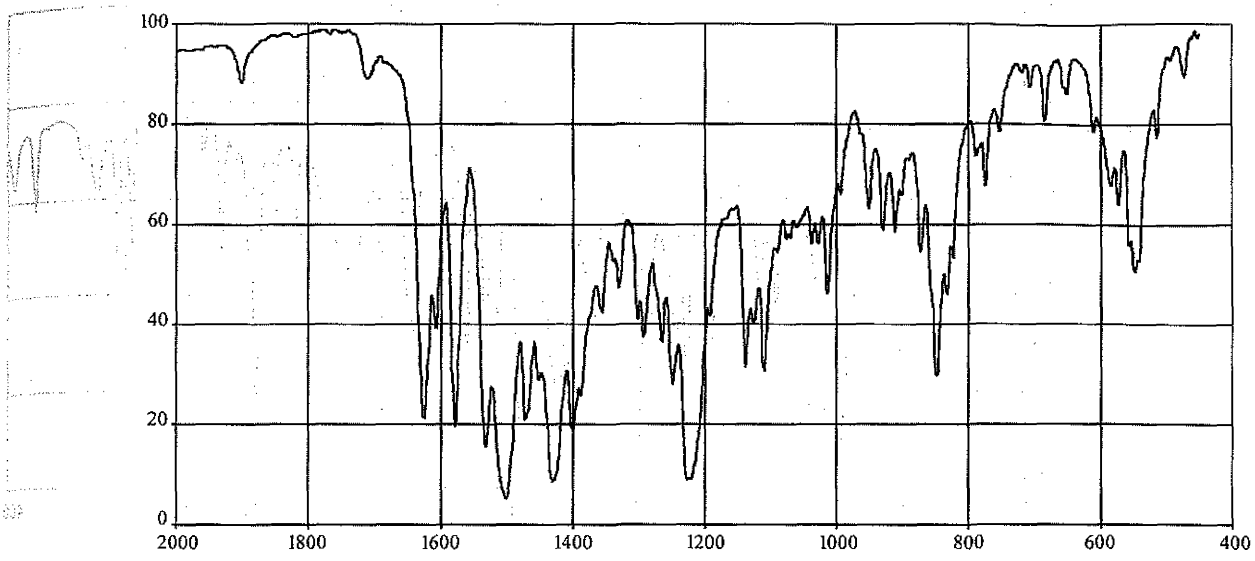
Galantamine Hydrobromide

FTIR

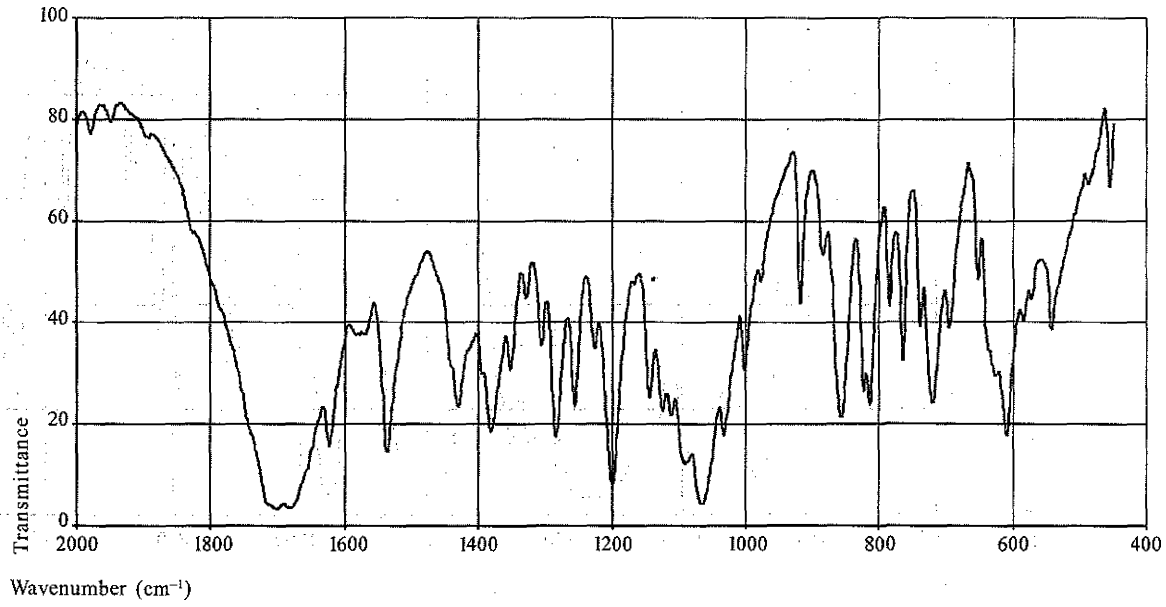
Phase: KBr disc



Gefitinib *FTIR* Phase: KBr disc



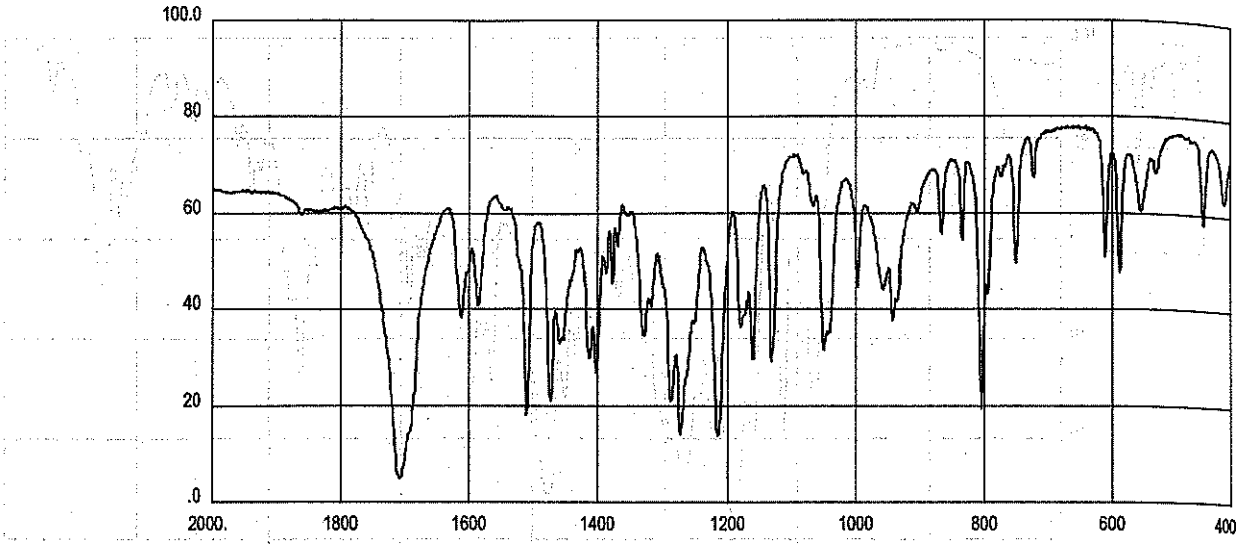
Gemcitabine Hydrochloride *FTIR* Phase: KBr disc



Gemfibrozil

FTIR

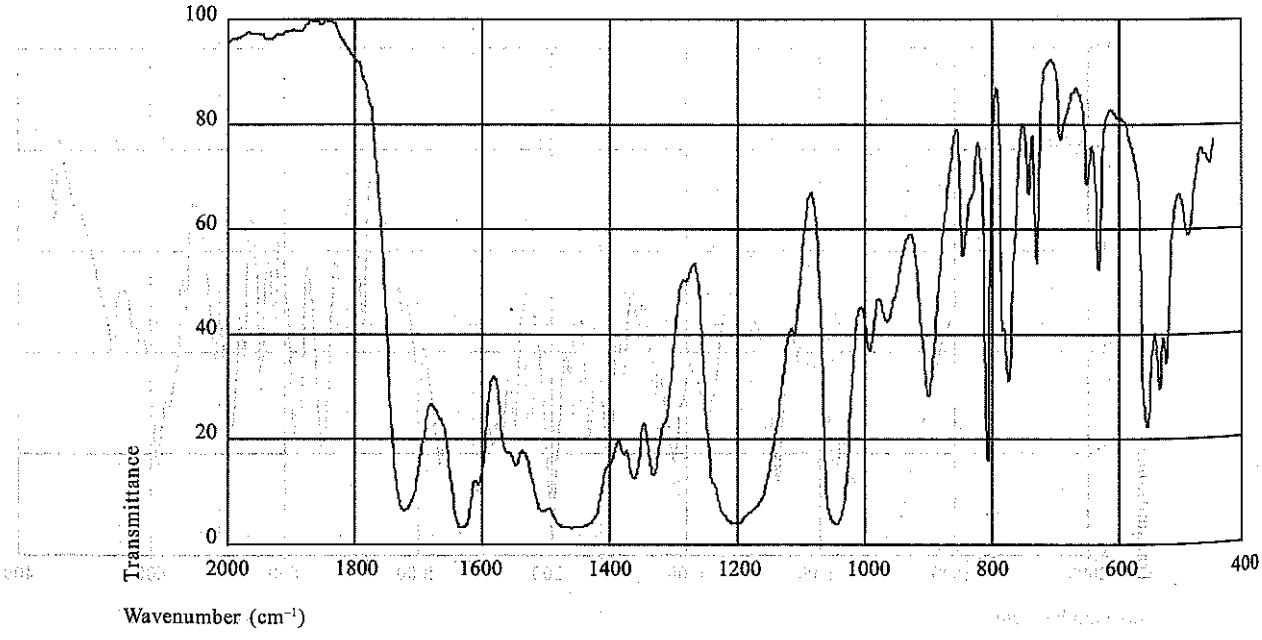
Phase: KBr disc



Gemifloxacin Mesylate

FTIR

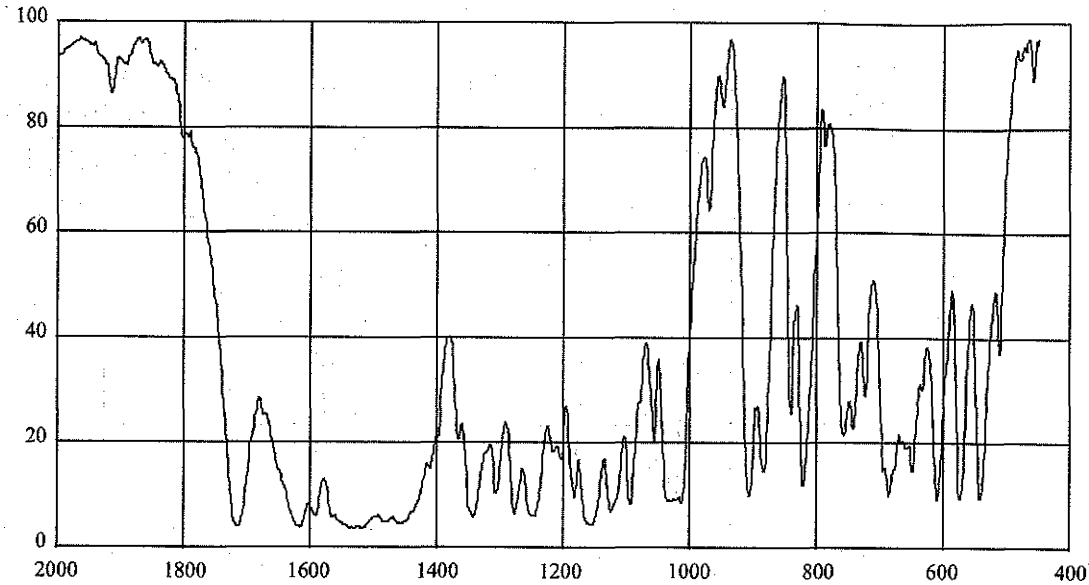
Phase: KBr disc



Glibenclamide

FTIR

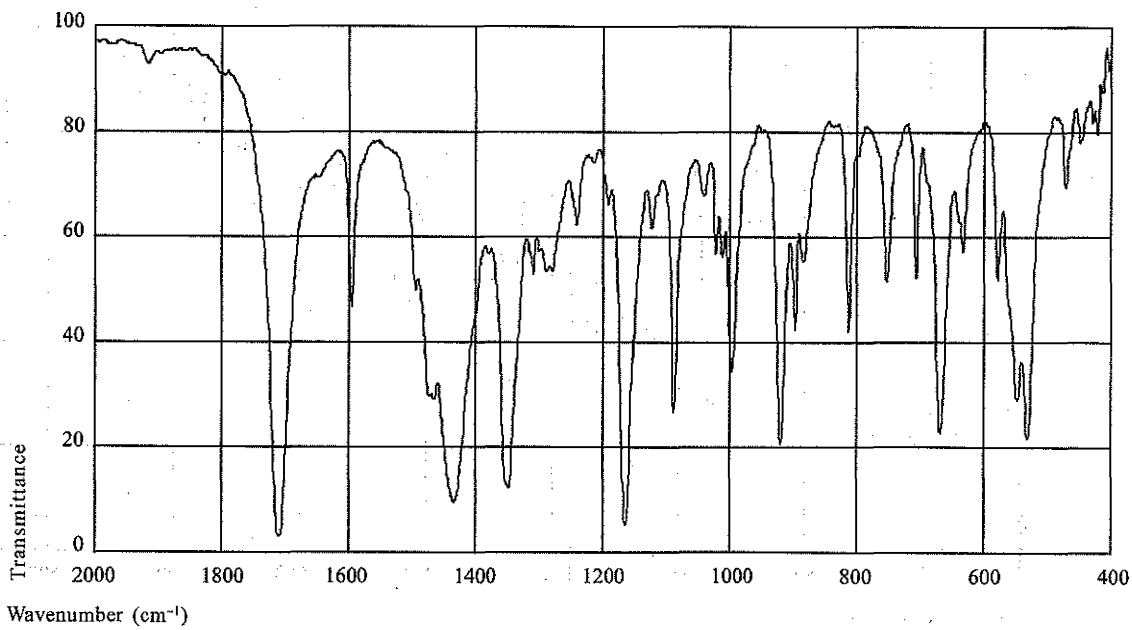
Phase: KBr disc



Gliclazide

FTIR

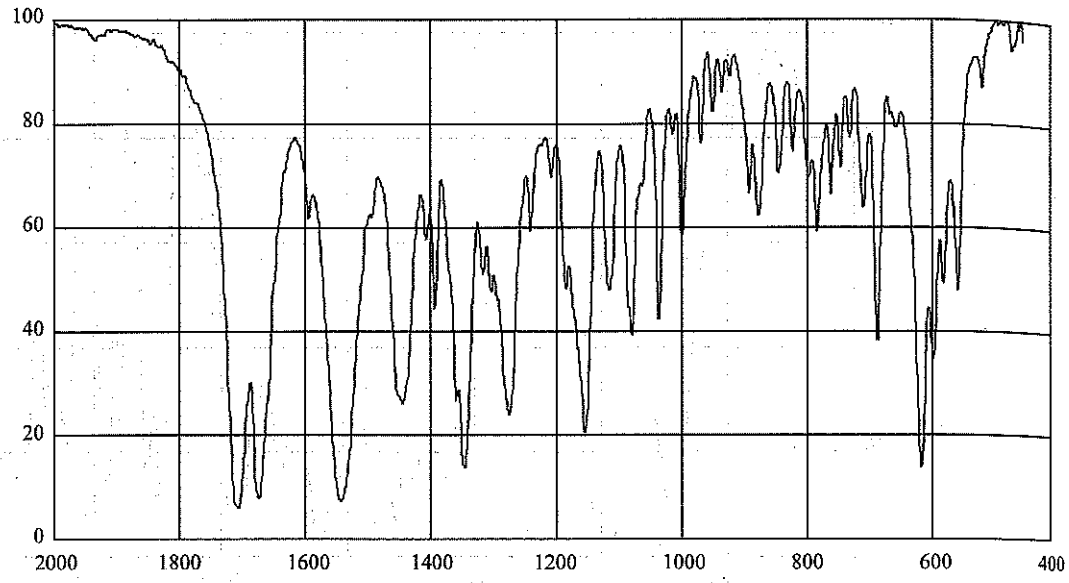
Phase: KBr disc



Glimepiride

FTIR

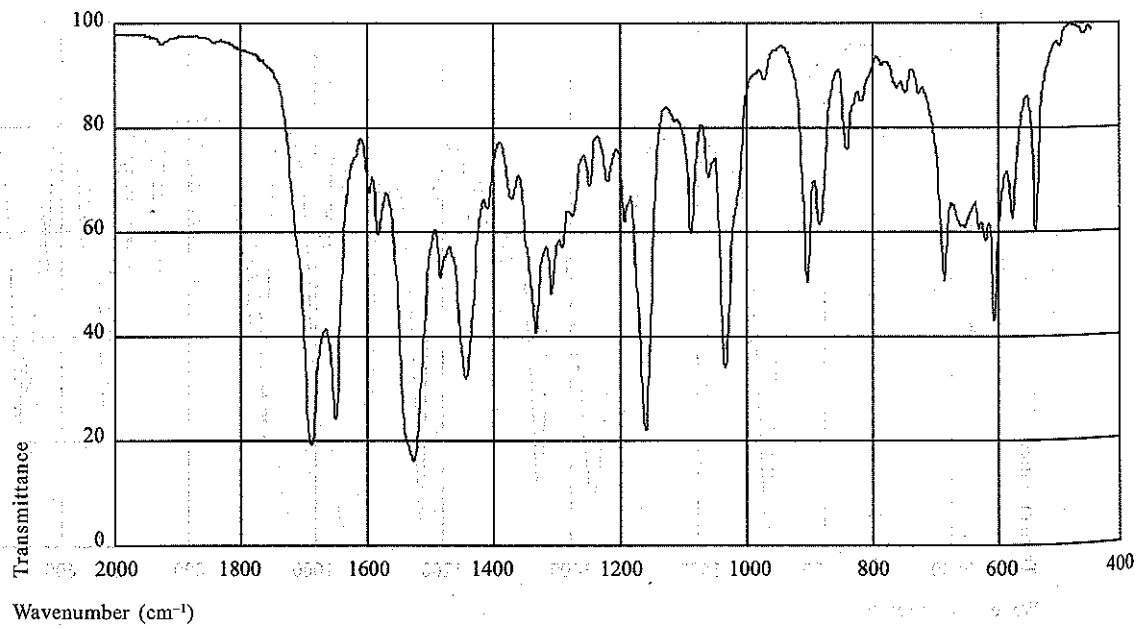
Phase: KBr disc



Glipizide

FTIR

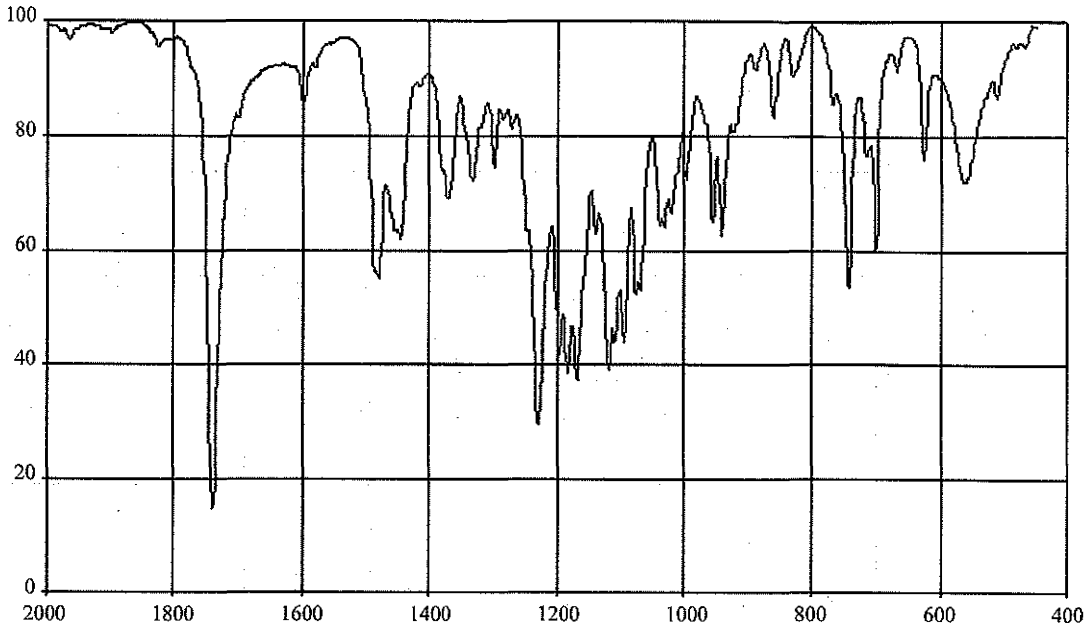
Phase: KBr disc



Glycopyrollate

FTIR

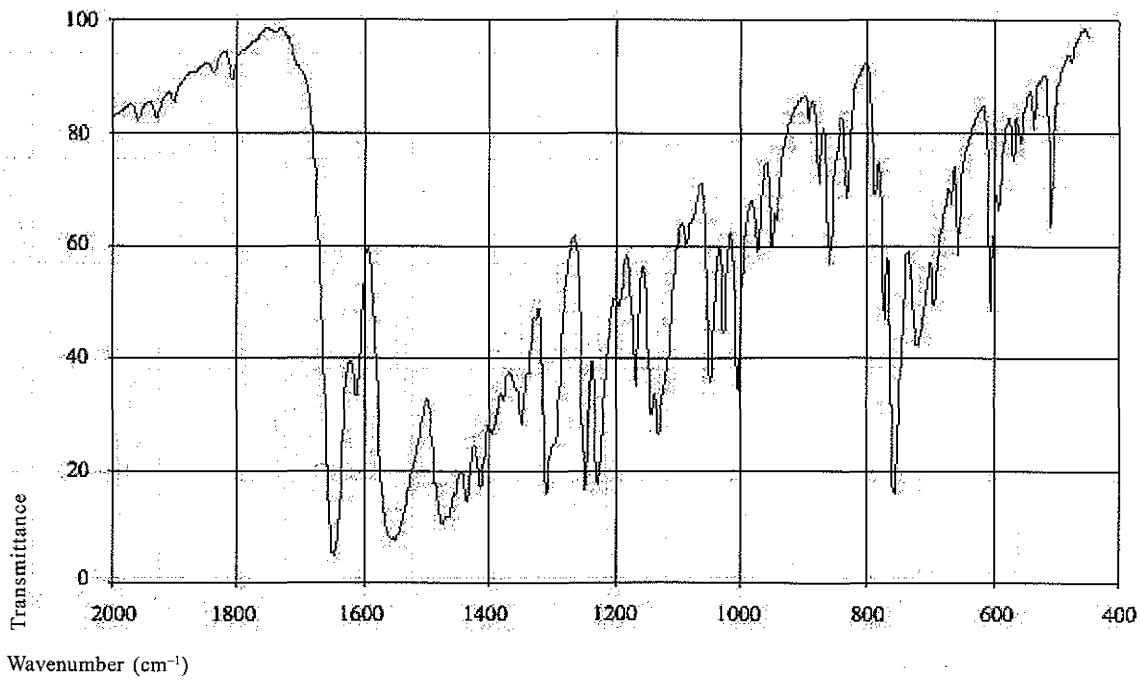
Phase: KBr disc



Granisetron Hydrochloride

FTIR

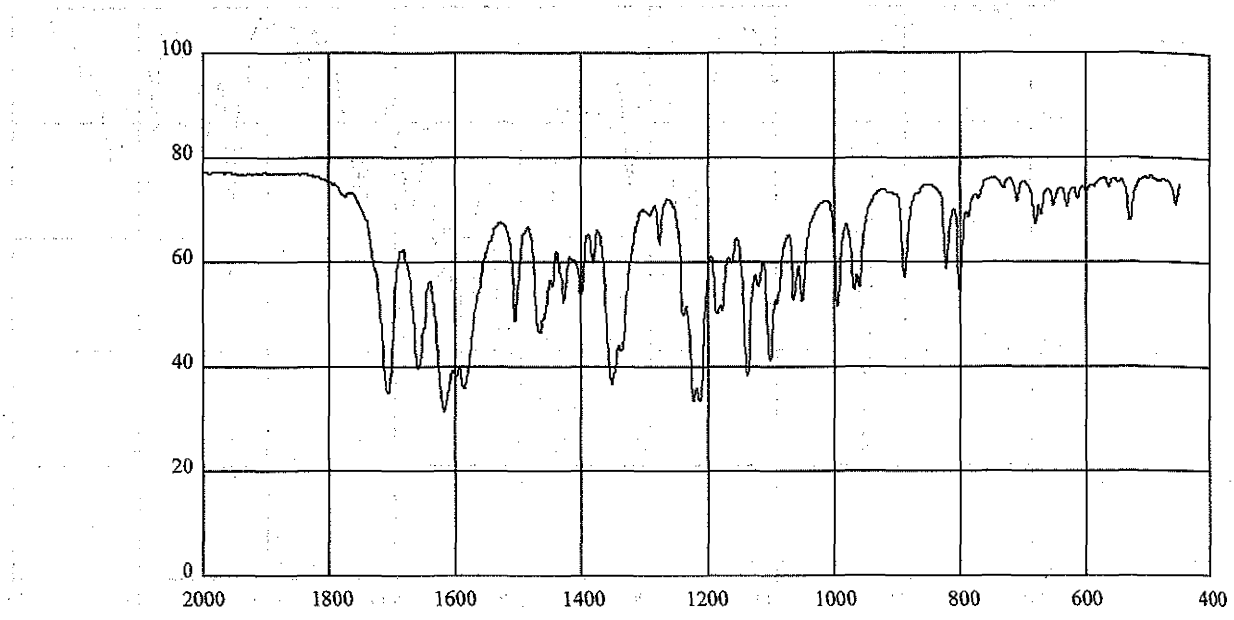
Phase: KBr disc



Griseofulvin

FTIR

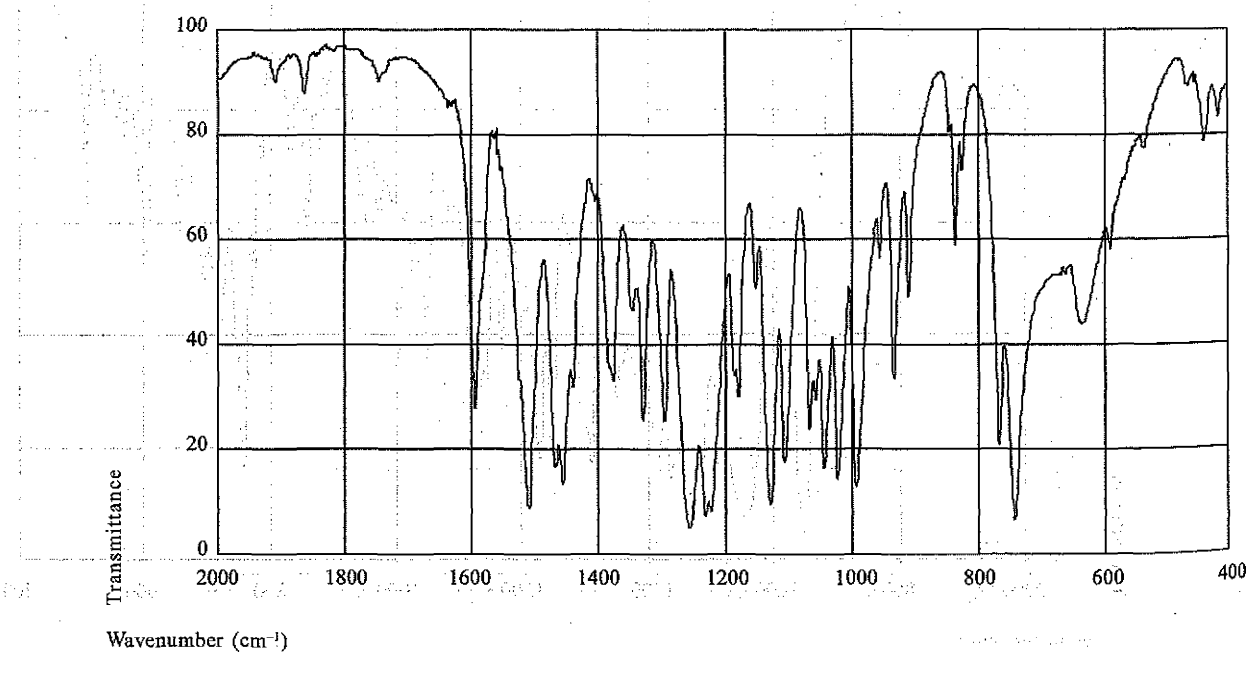
Phase: KBr disc



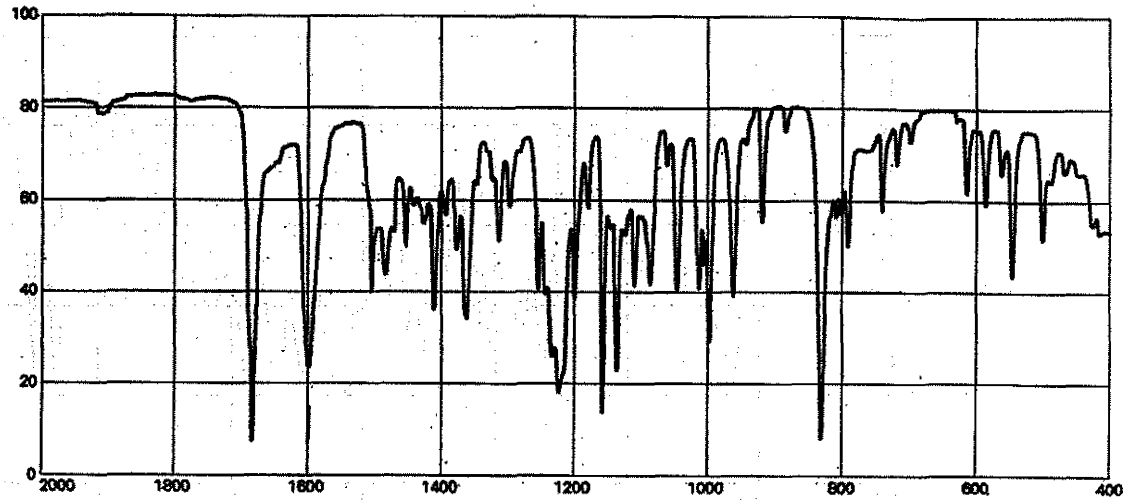
Guaiphenesin

FTIR

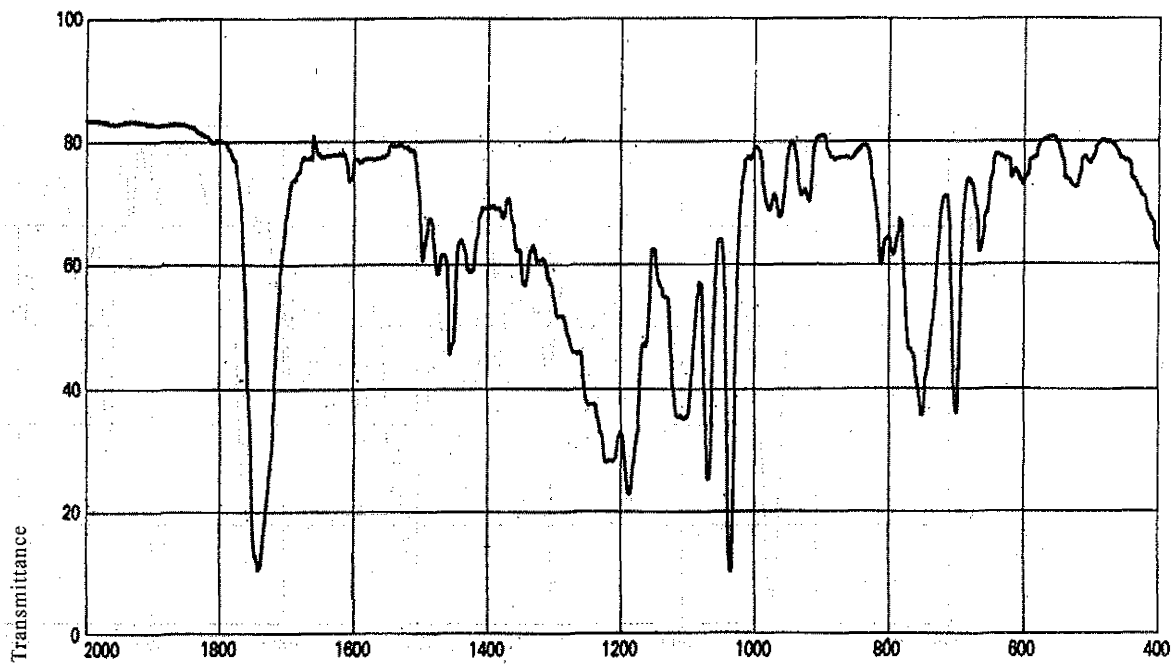
Phase: KBr disc



Haloperidol *Dispersive IR* Phase: KCl disc



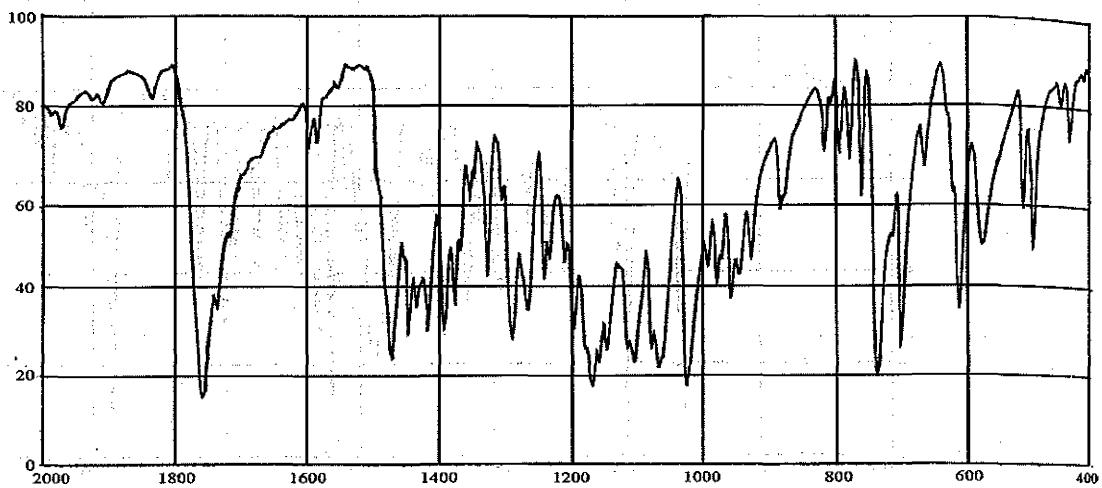
Homatropine *Dispersive IR* Phase: Thin film



Homatropine Hydrobromide

FTIR

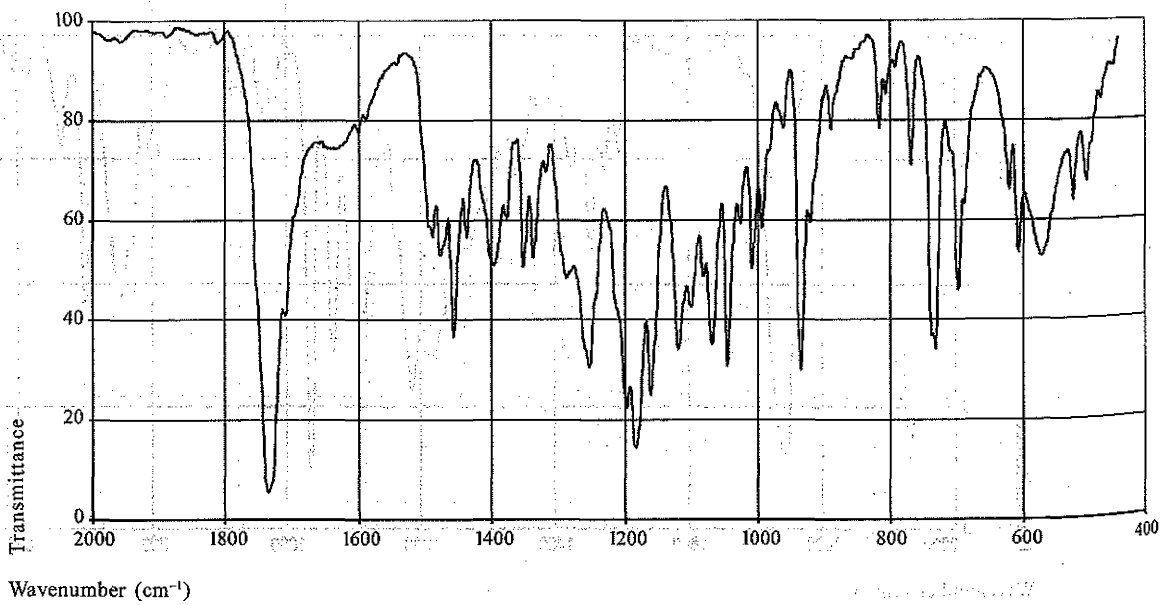
Phase: KBr disc



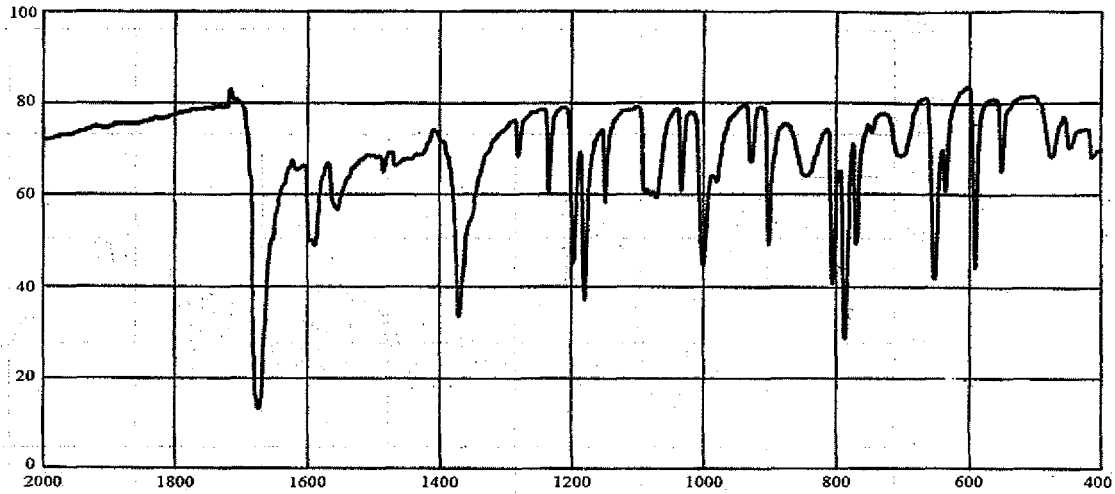
Homatropine Methylbromide

FTIR

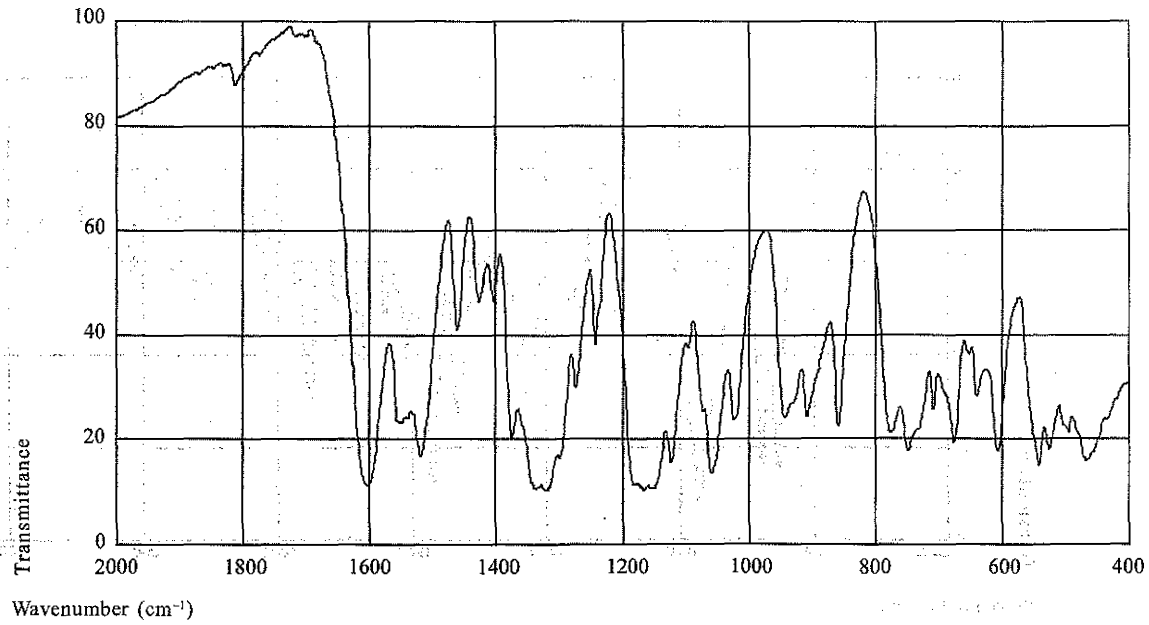
Phase: KBr disc



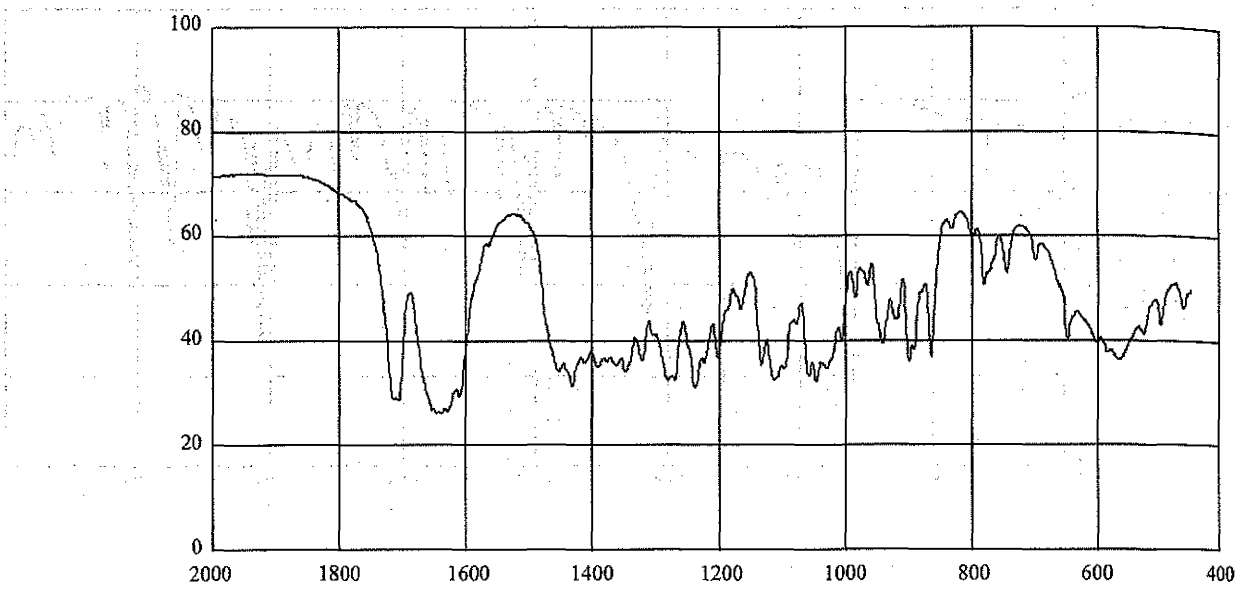
Hydralazine Hydrochloride *Dispersive IR* Phase: KCl disc



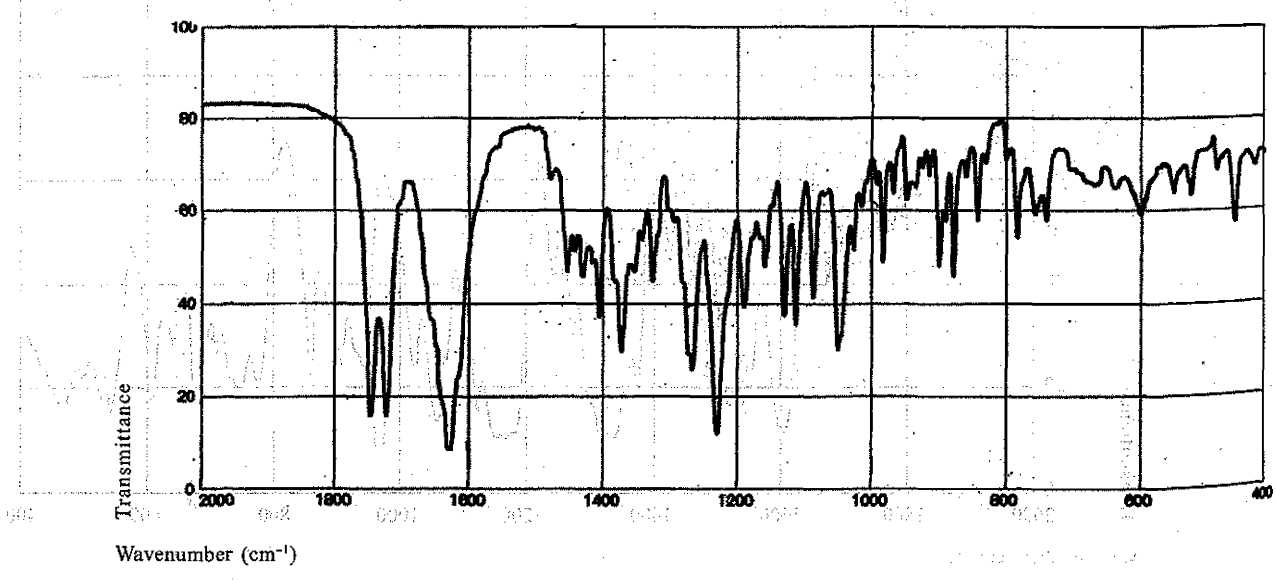
Hydrochlorothiazide *FTIR* Phase: KBr disc



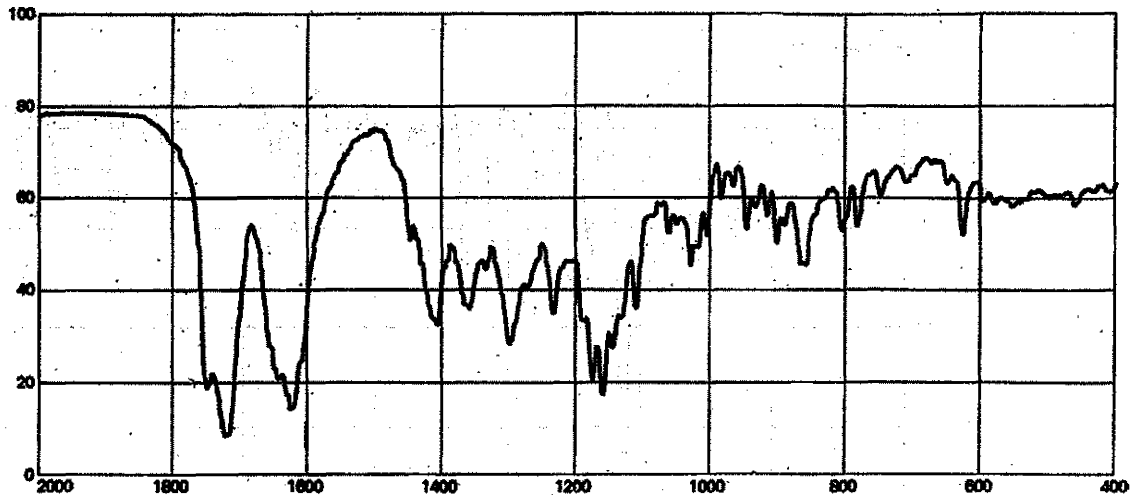
Hydrocortisone *FTIR* Phase: KBr disc



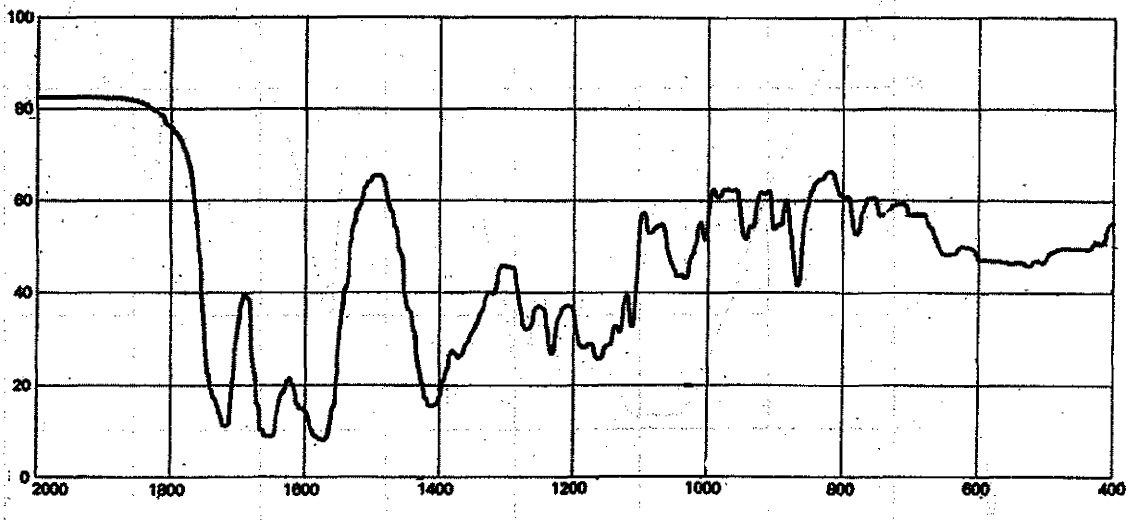
Hydrocortisone Acetate *Dispersive IR* Phase: KBr disc



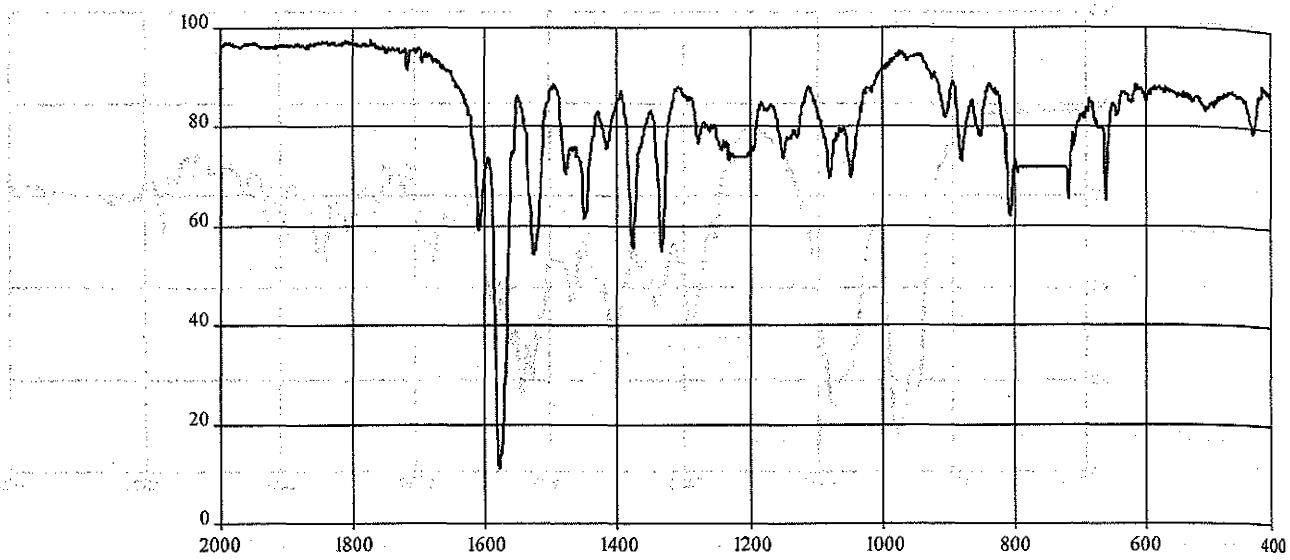
Hydrocortisone Hemisuccinate *Dispersive IR* Phase: KBr disc



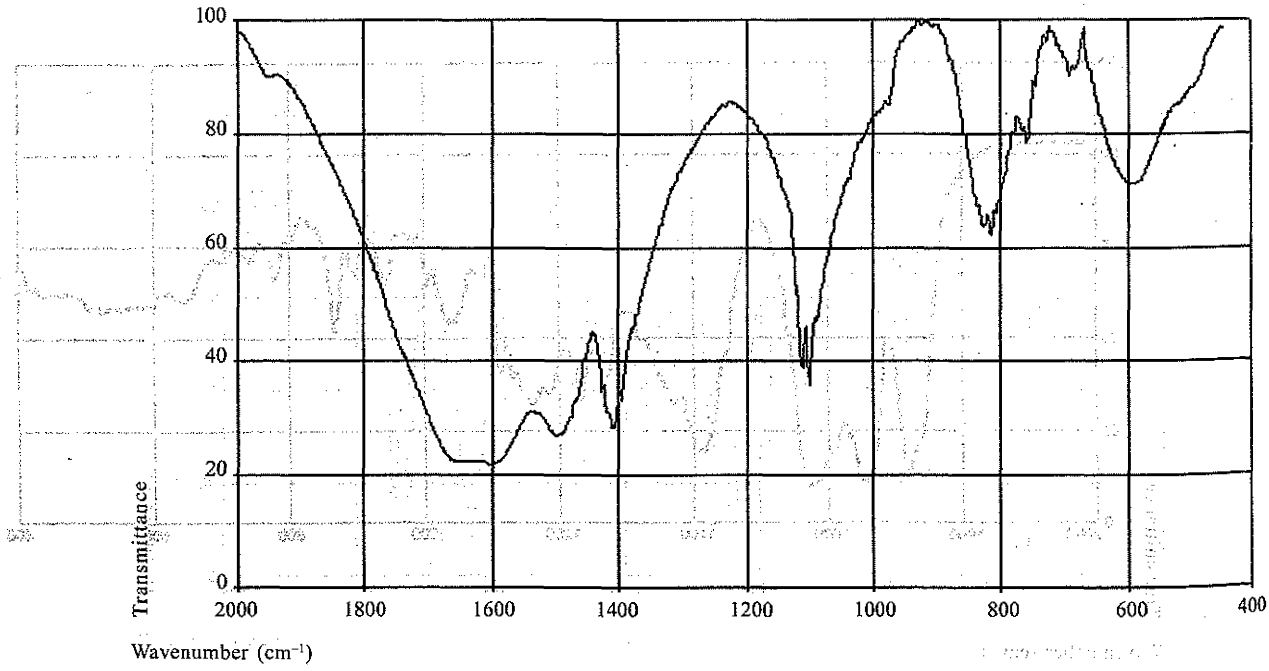
Hydrocortisone Sodium Succinate *Dispersive IR* Phase: KBr disc



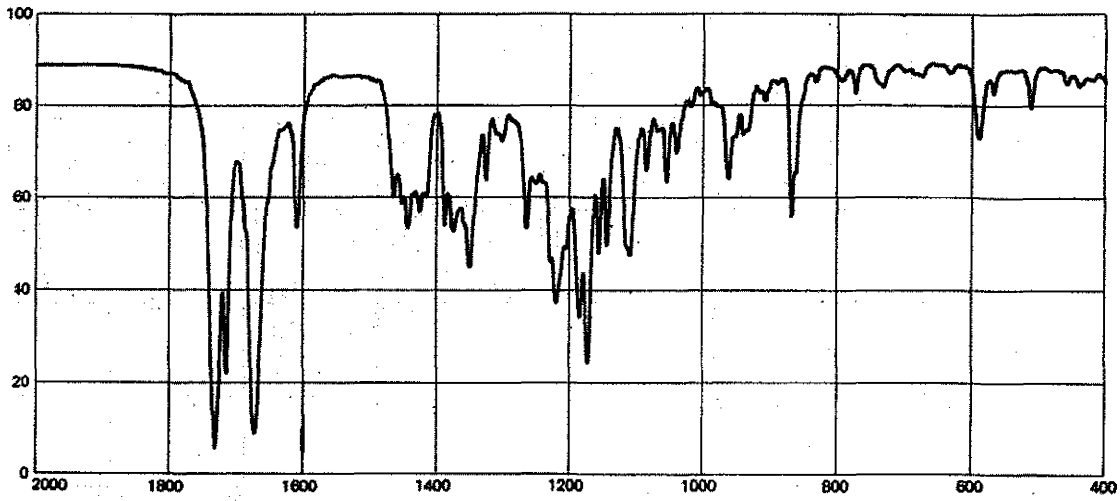
Hydroxychloroquine *Dispersive IR* Phase: KBr disc



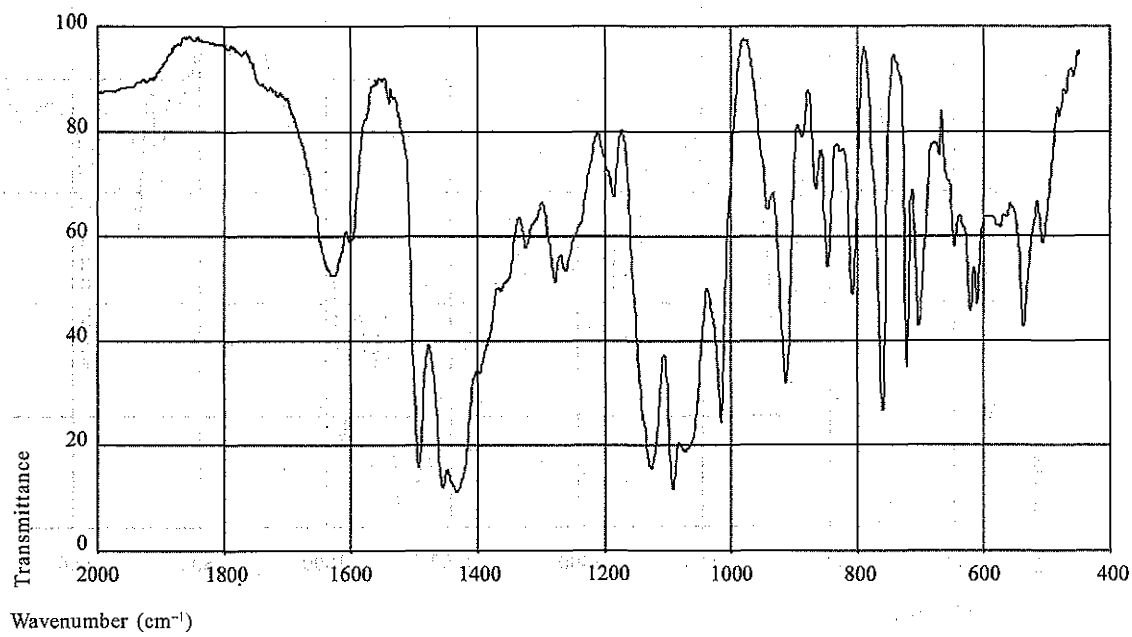
Hydroxylurea *FTIR* Phase: KBr disc



Hydroxyprogesterone Hexanoate *Dispersive IR* Phase: KBr disc

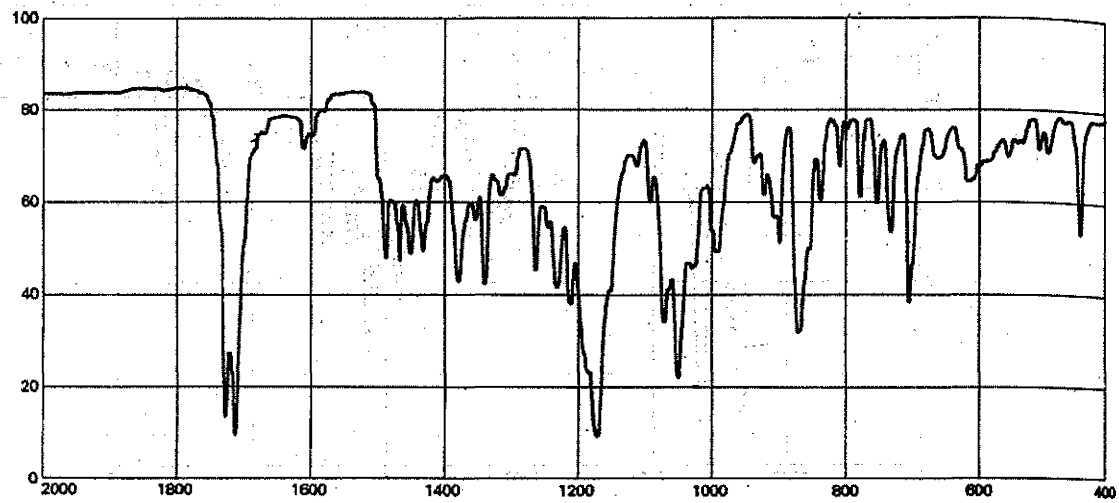


Hydroxyzine Hydrochloride *FTIR* Phase: KBr disc



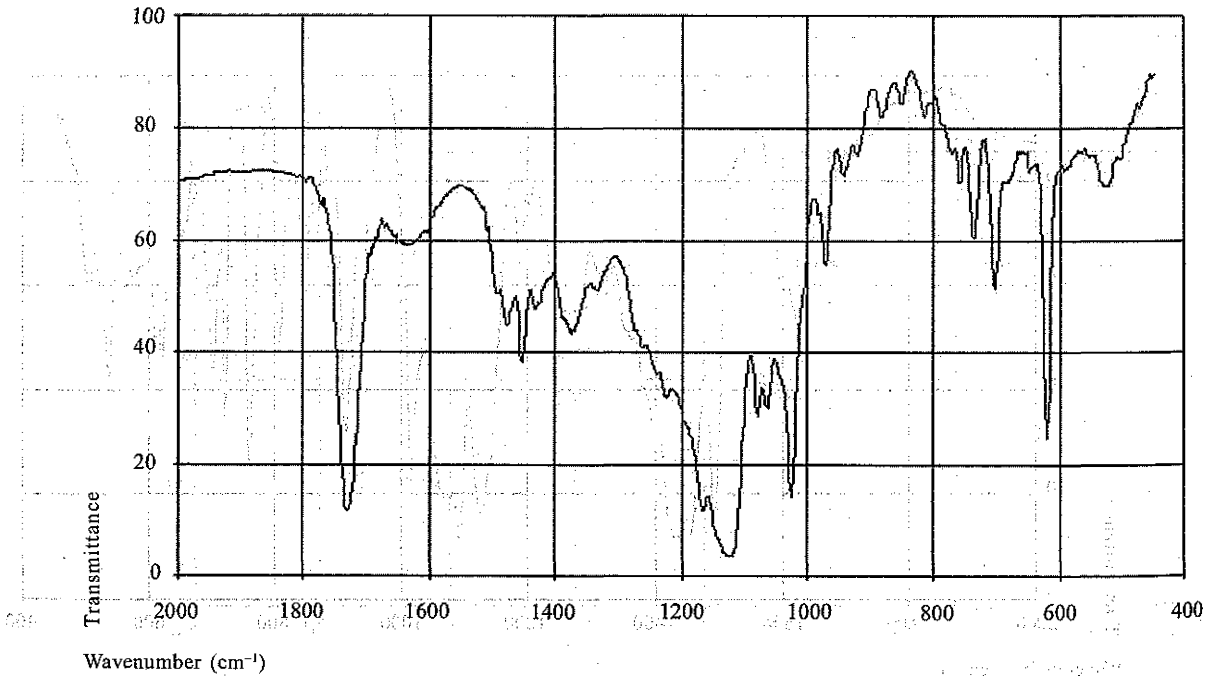
Hyoscine Butylbromide

Dispersive IR Phase: KBr disc

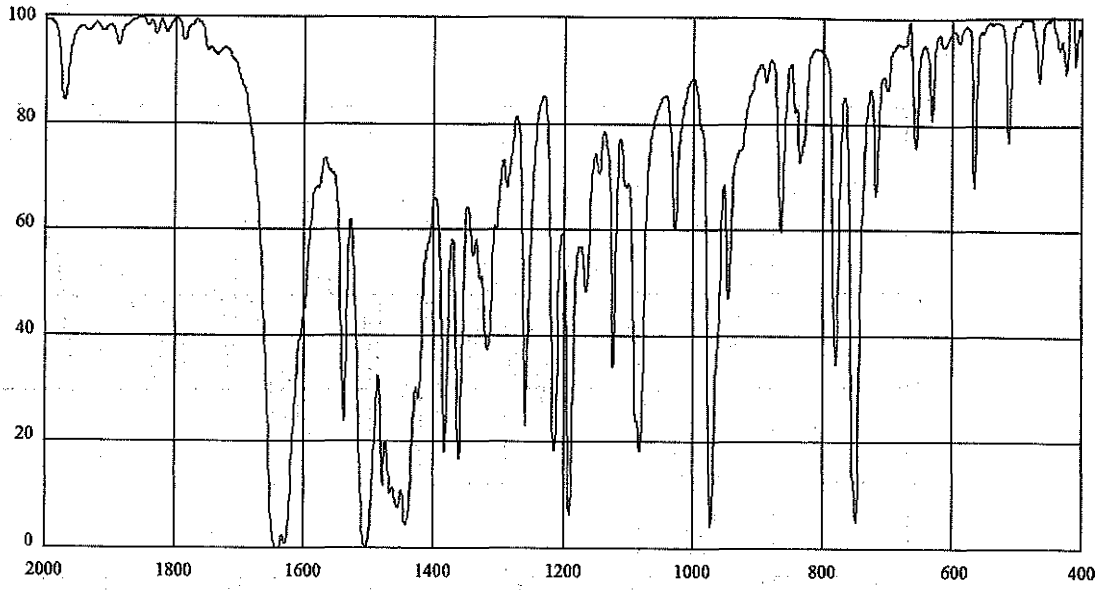


Hyoscyamine Sulphate

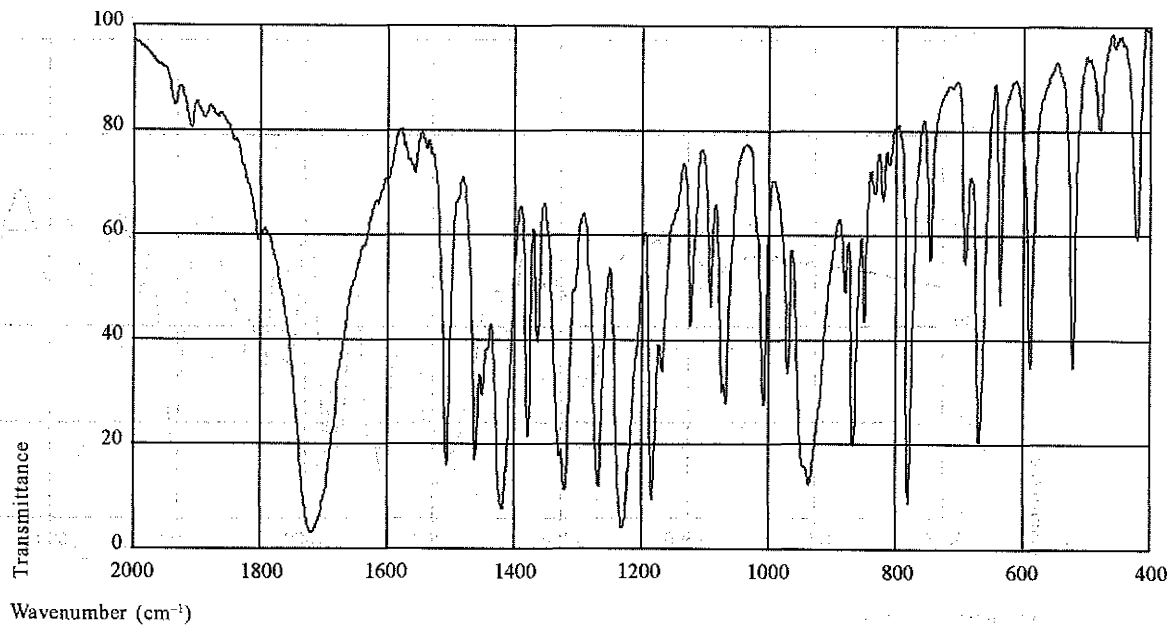
FTIR Phase: KBr disc



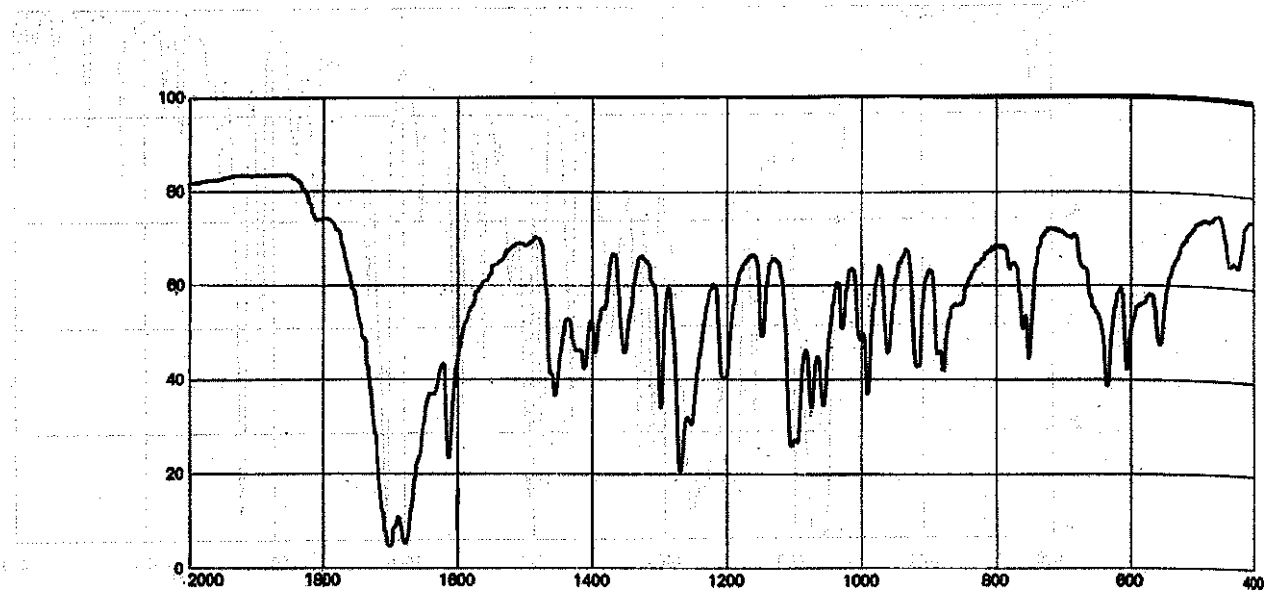
Ibudilast *FTIR* Phase: KBr disc



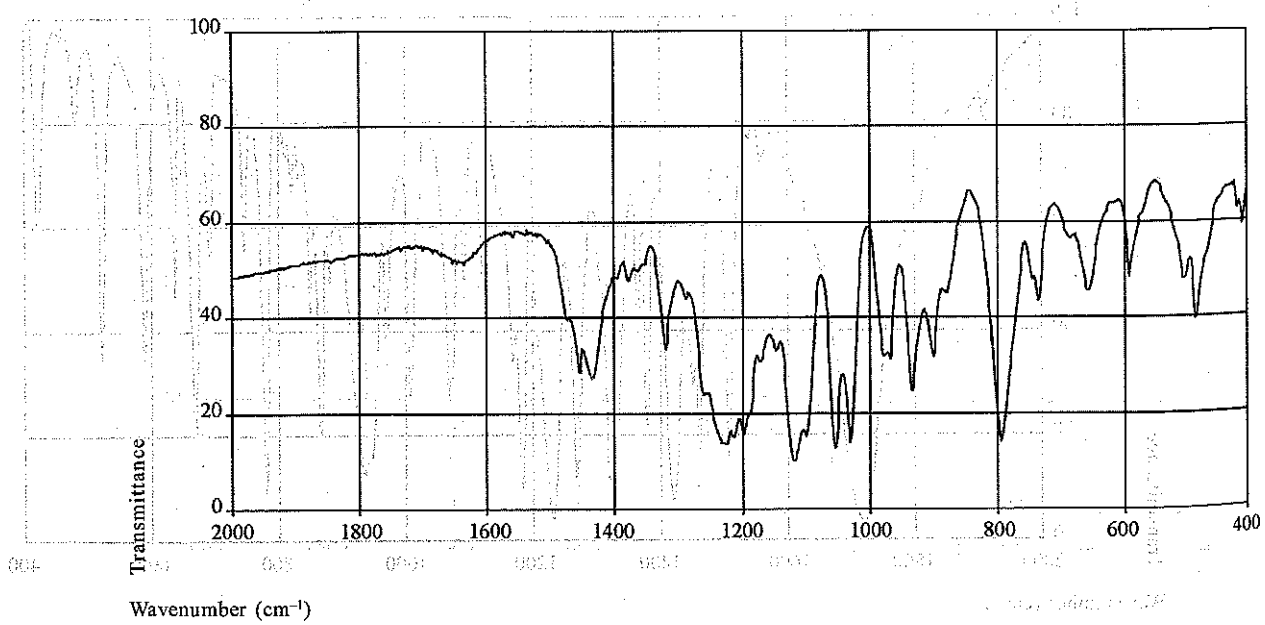
Ibuprofen *FTIR* Phase: KBr disc



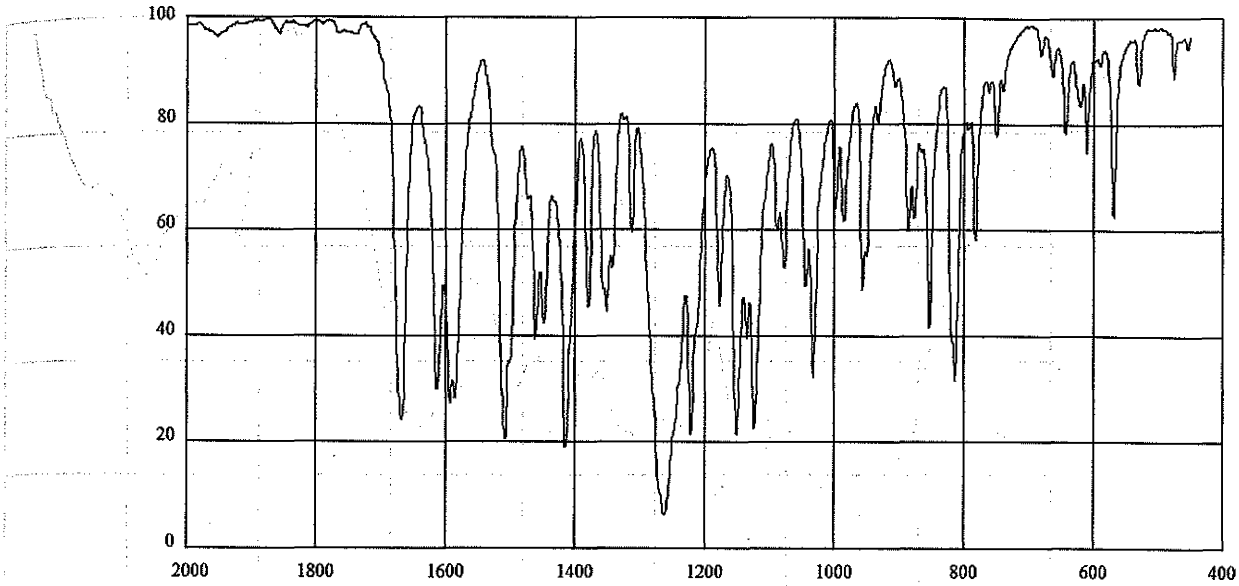
Idoxuridine *Dispersive IR* Phase: KBr disc



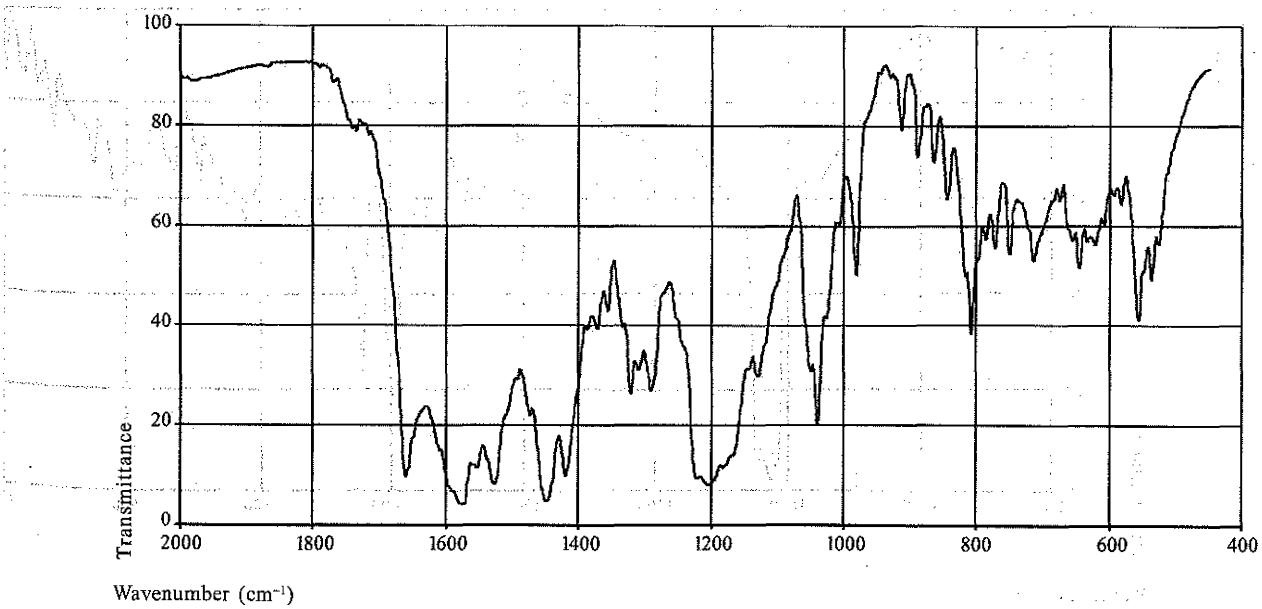
Ifosfamide *FTIR* Phase: KBr disc



Iloperidone *FTIR* Phase: KBr disc



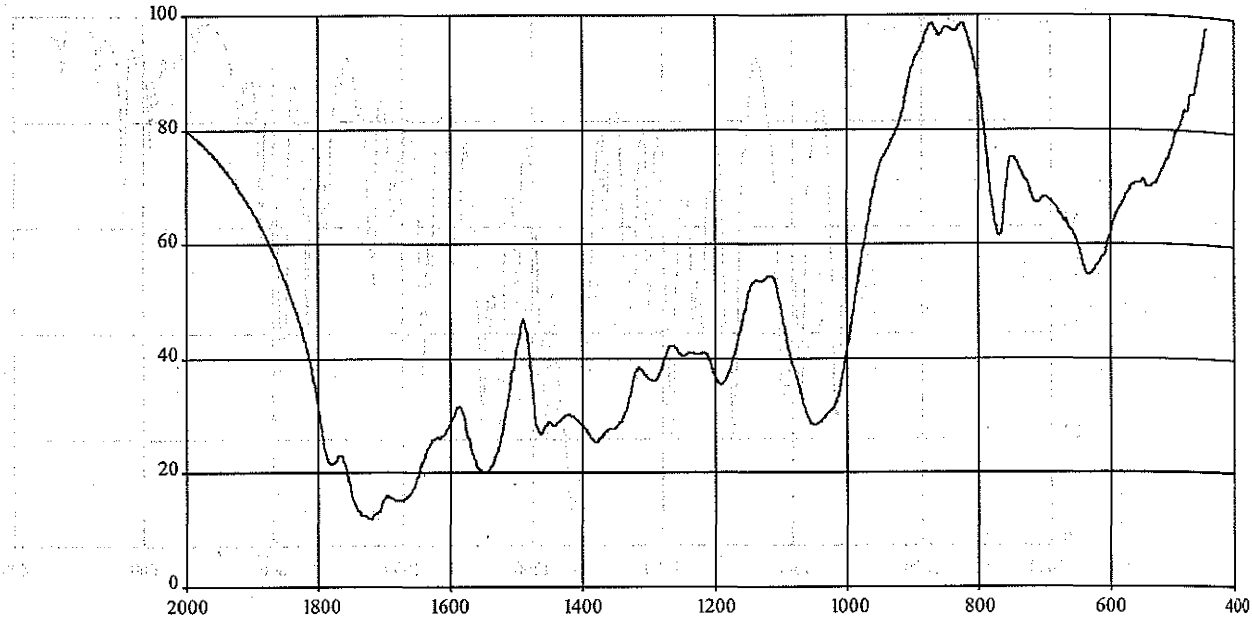
Imatinib Mesylate *FTIR* Phase: KBr disc



Imidurea

FTIR

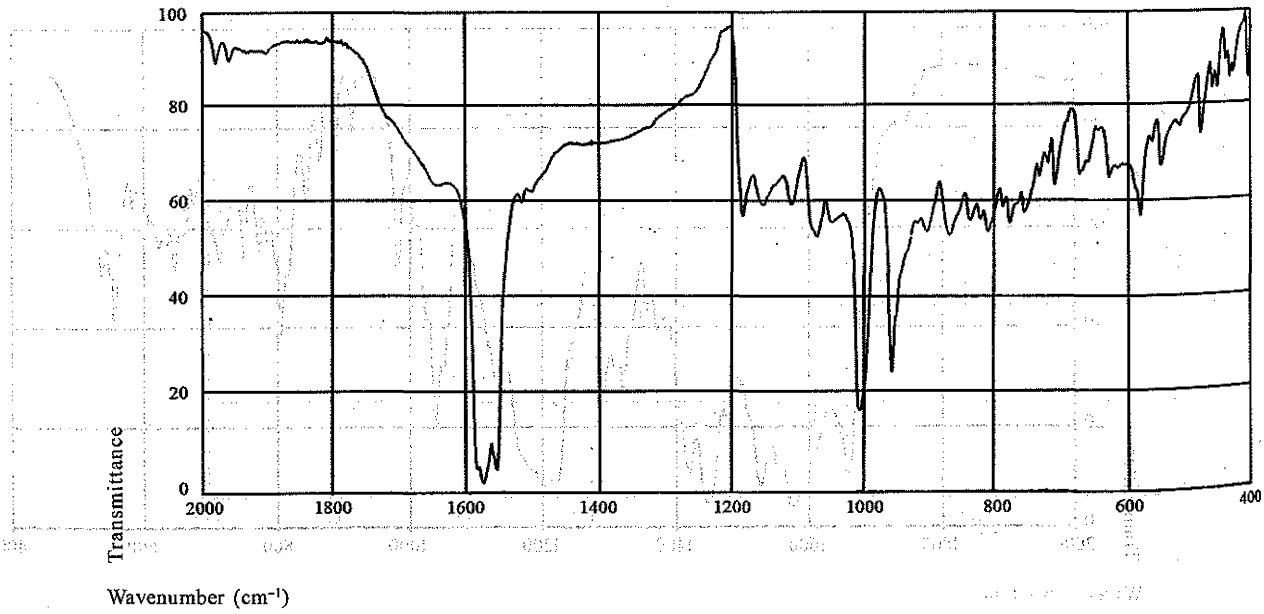
Phase: KBr disc



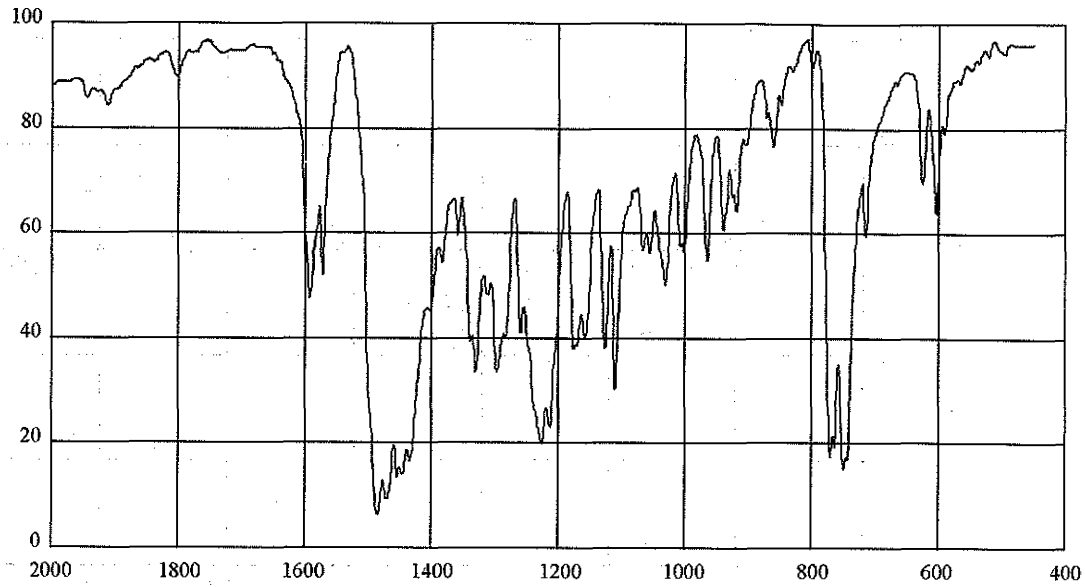
Imipenem Monohydrate

FTIR

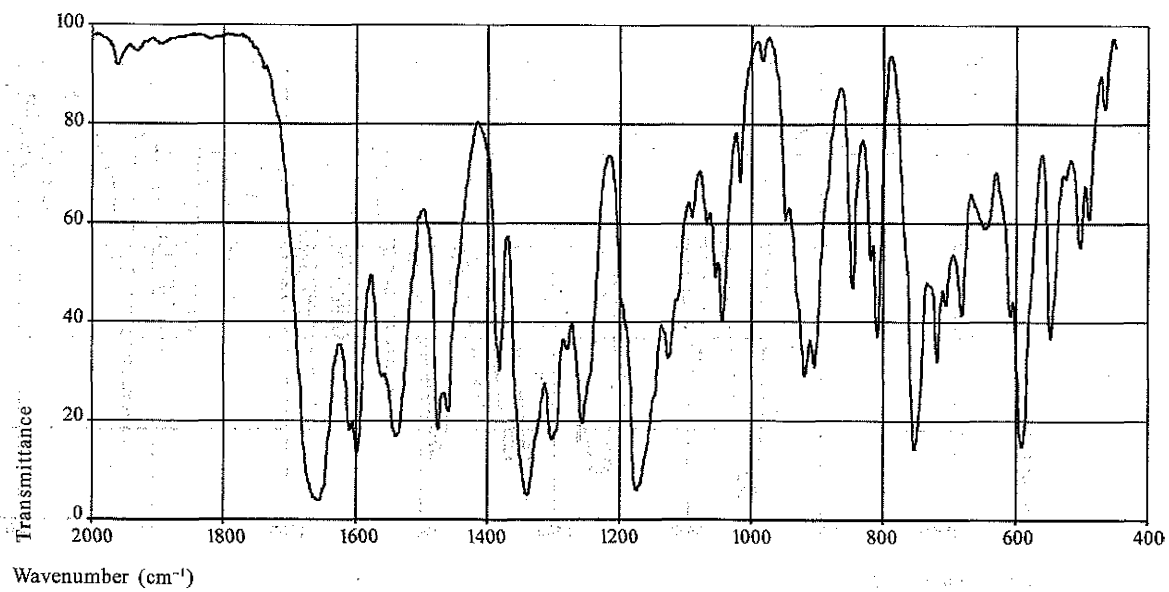
Phase: Liquid paraffin mull



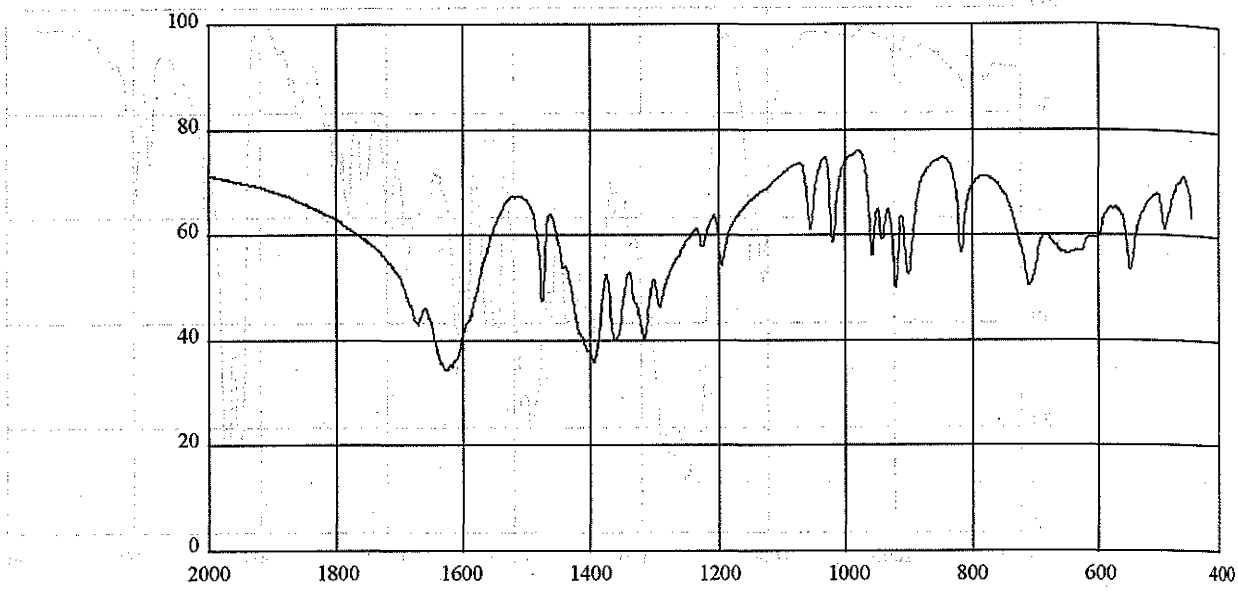
Imipramine Hydrochloride *FTIR* Phase: KBr disc



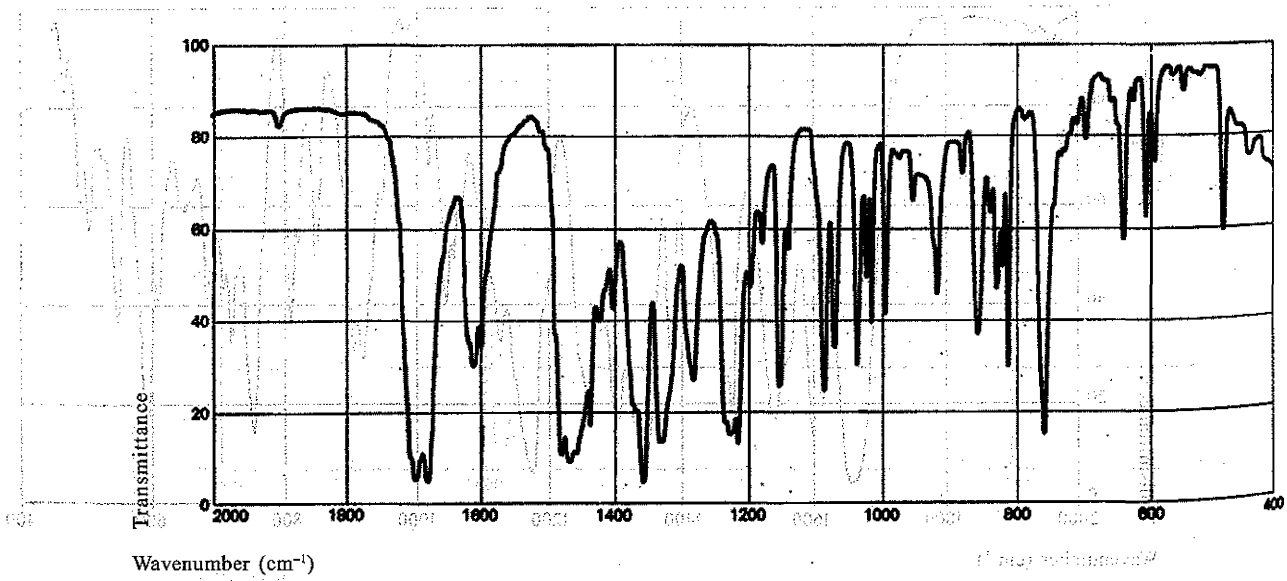
Indapamide *FTIR* Phase: KBr disc



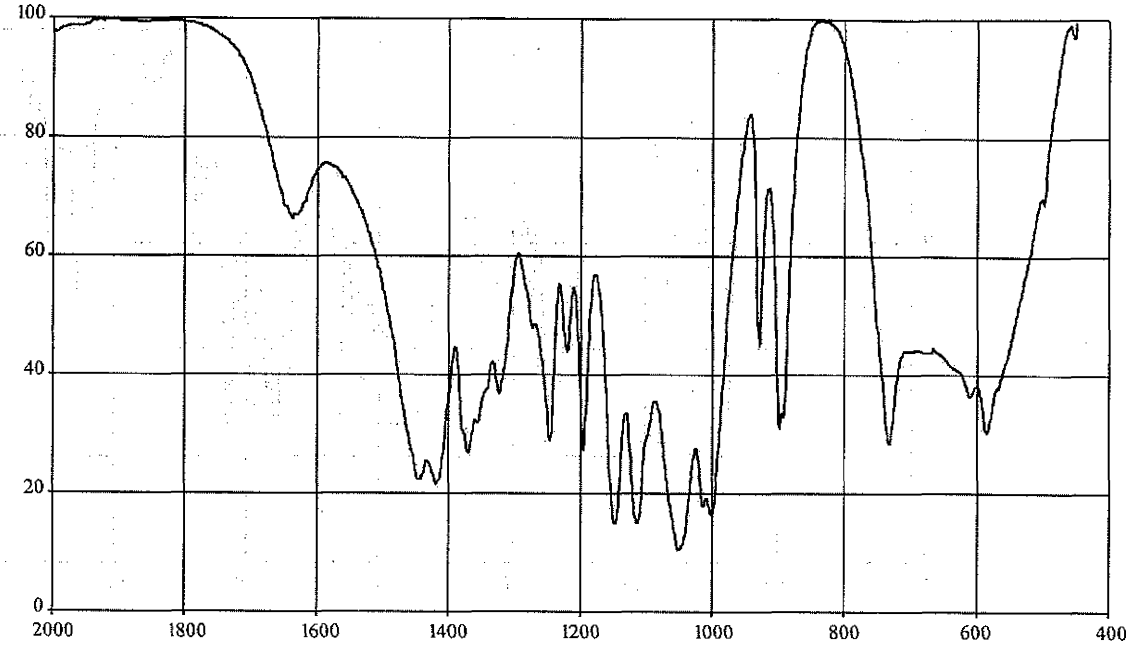
Indinavir Sulphate *FTIR* Phase: KBr disc



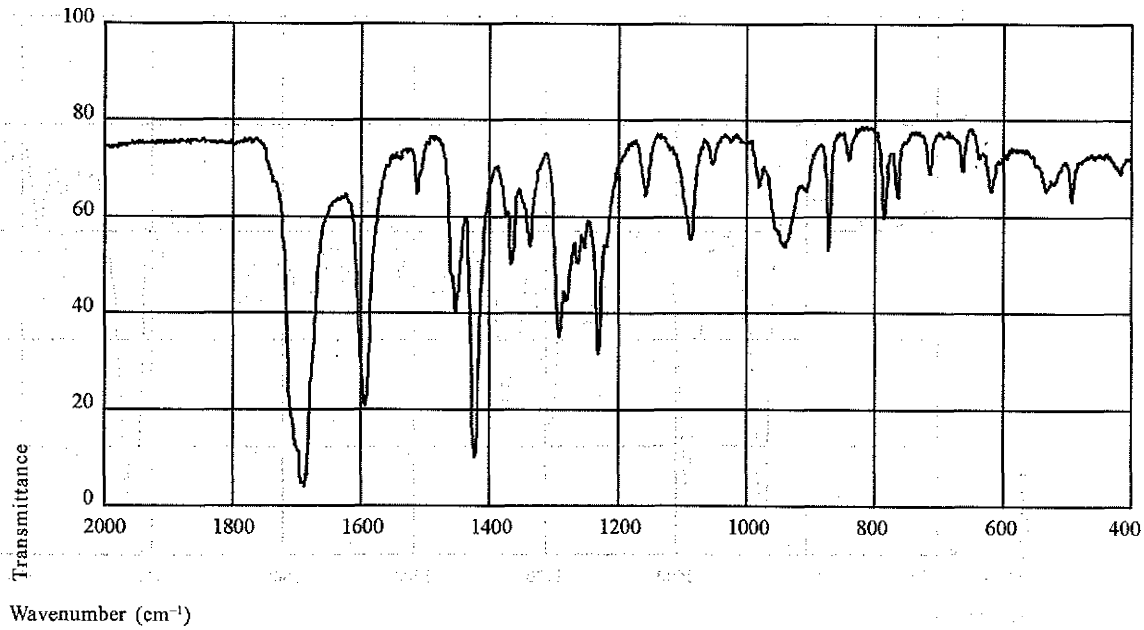
Indomethacin *Dispersive IR* Phase: Liquid paraffin mull



Inositol *Dispersive IR* Phase: Liquid paraffin mull



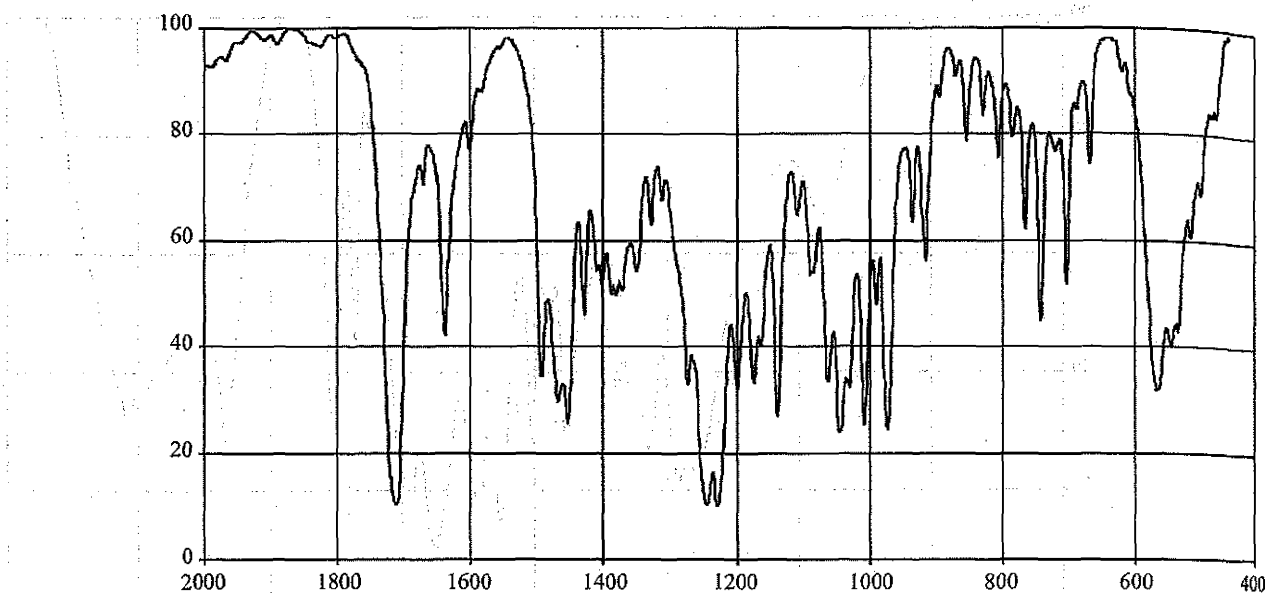
Iopanoic Acid *FTIR* Phase: KBr disc



Ipratropium Bromide

FTIR

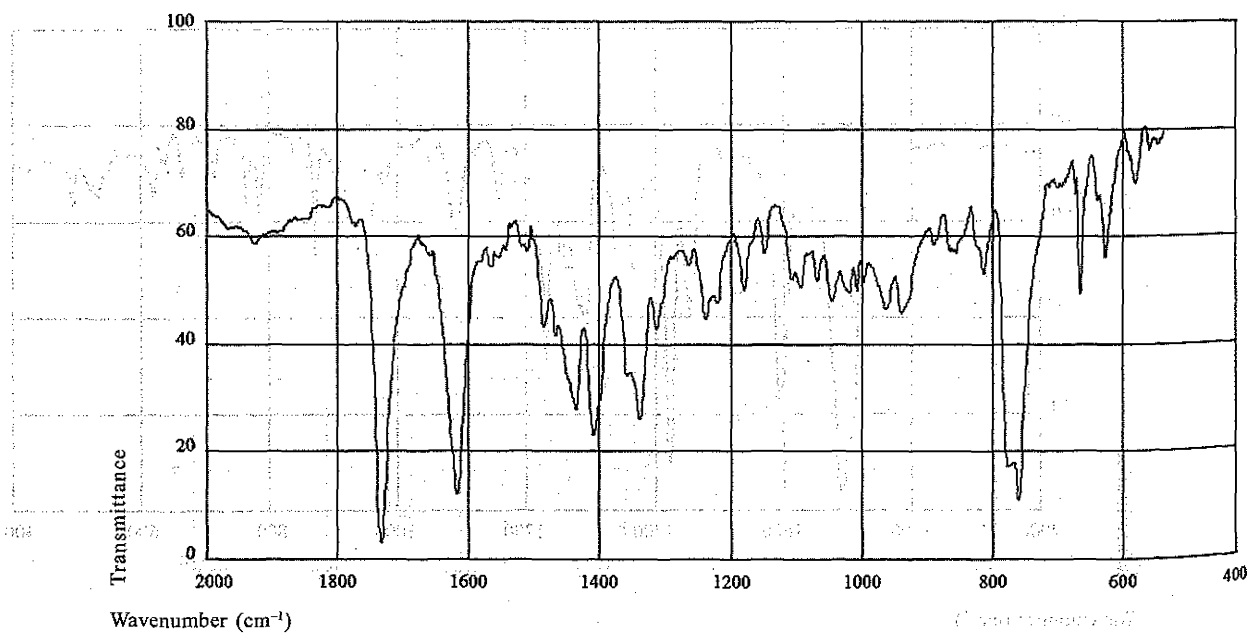
Phase: KBr disc



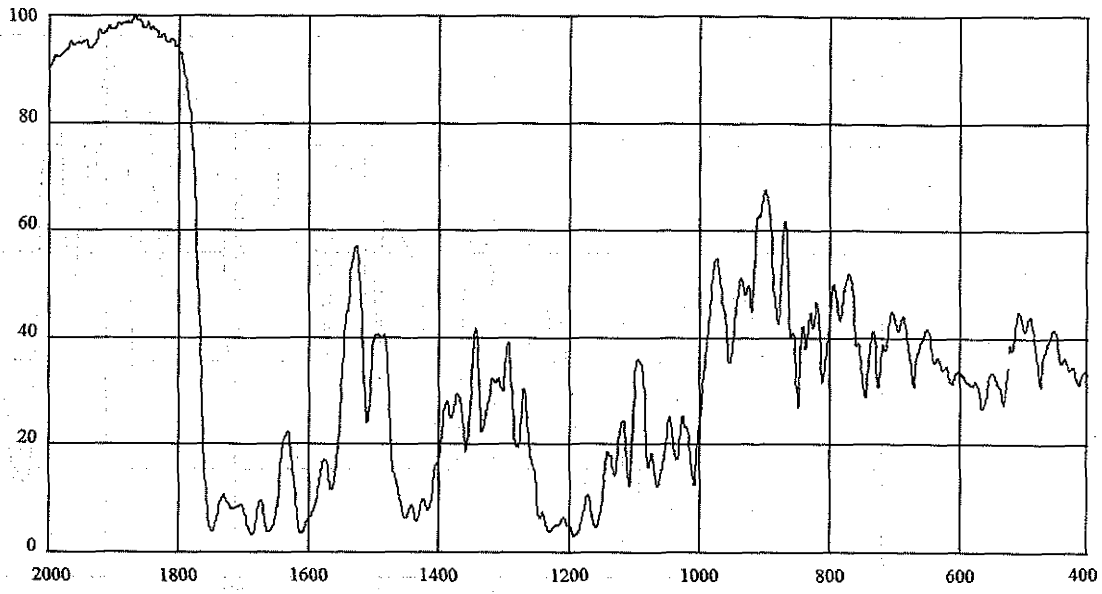
Irbesartan

FTIR

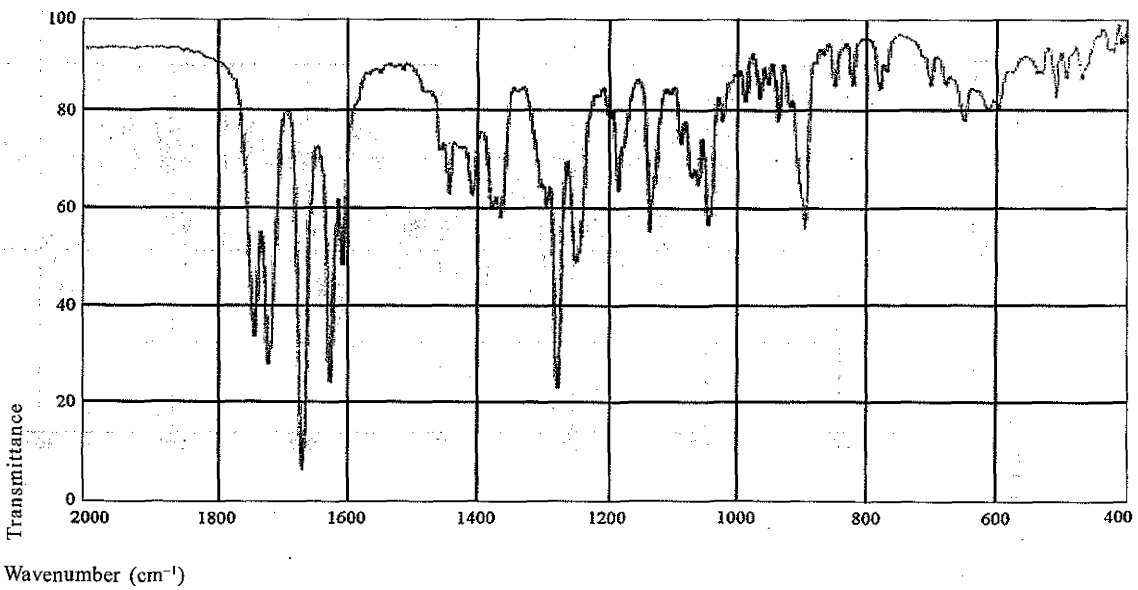
Phase: KBr disc



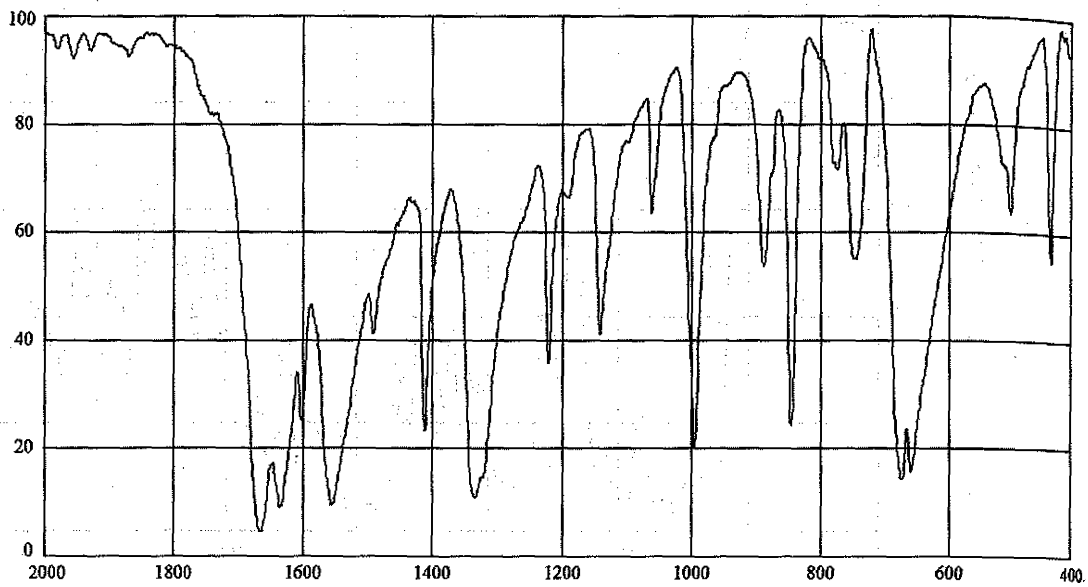
Irinotecan Hydrochloride Trihydrate *FTIR* Phase: KBr disc



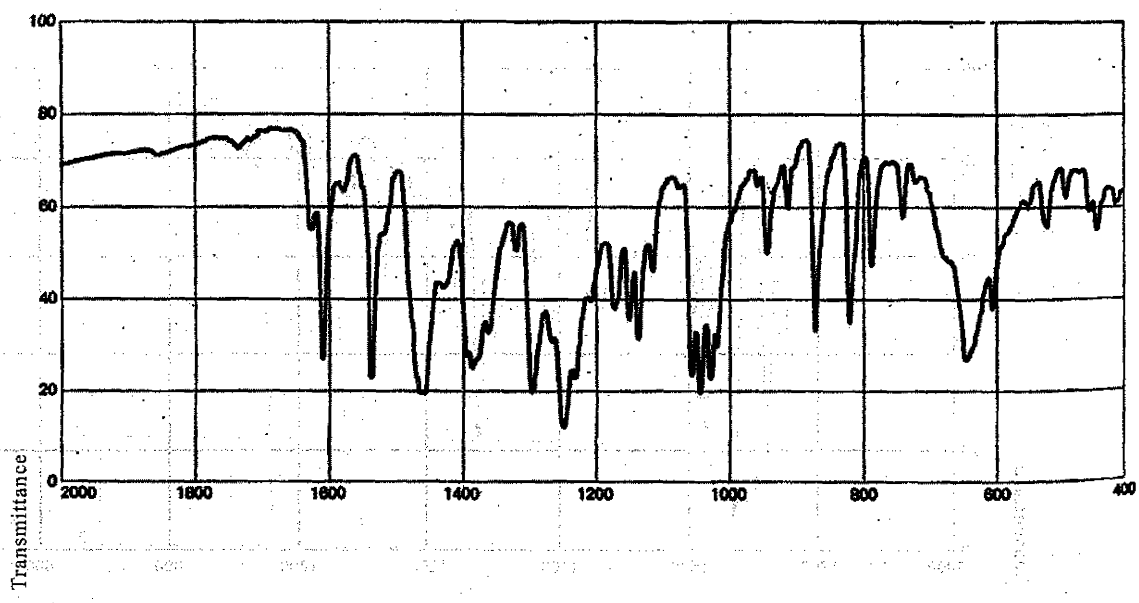
Isoflupredone Acetate *FTIR* Phase: KBr disc



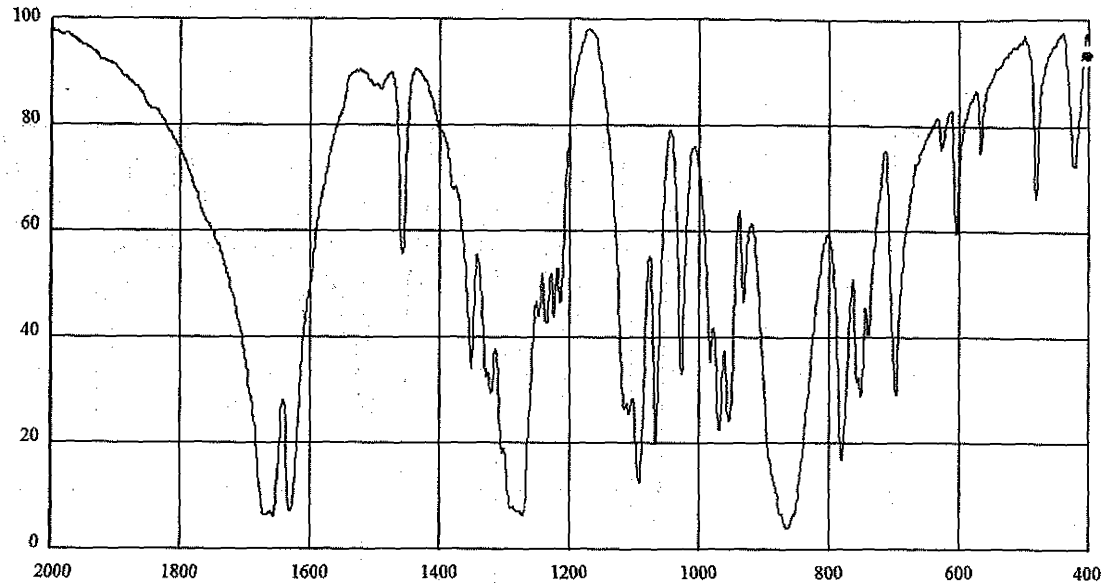
Isoniazid *Dispersive IR* Phase: KBr disc



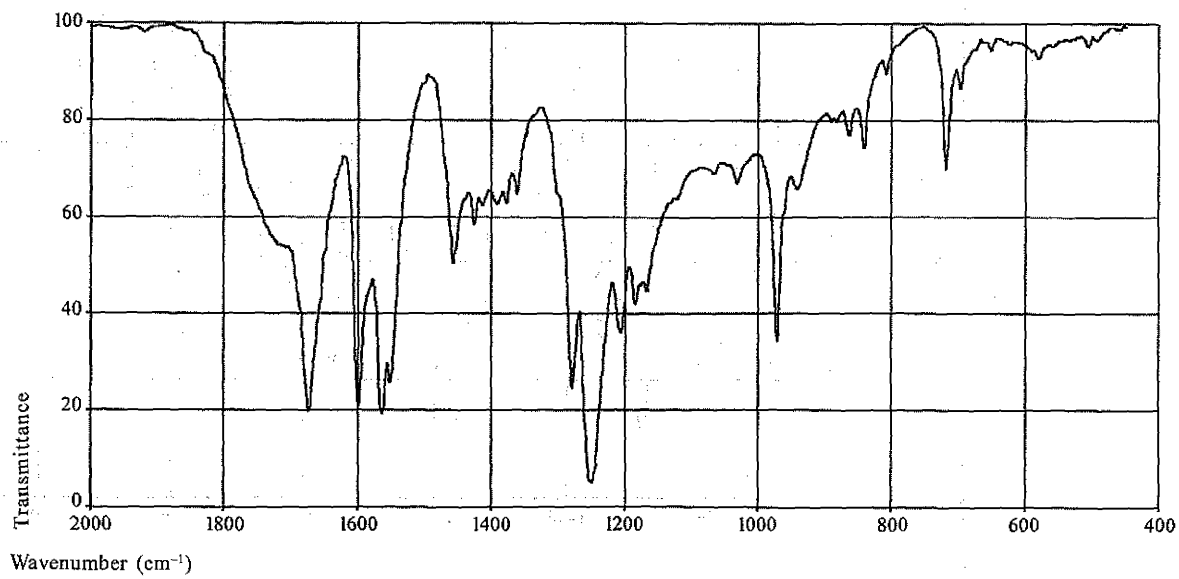
Isoprenaline Hydrochloride *Dispersive IR* Phase: KCl disc



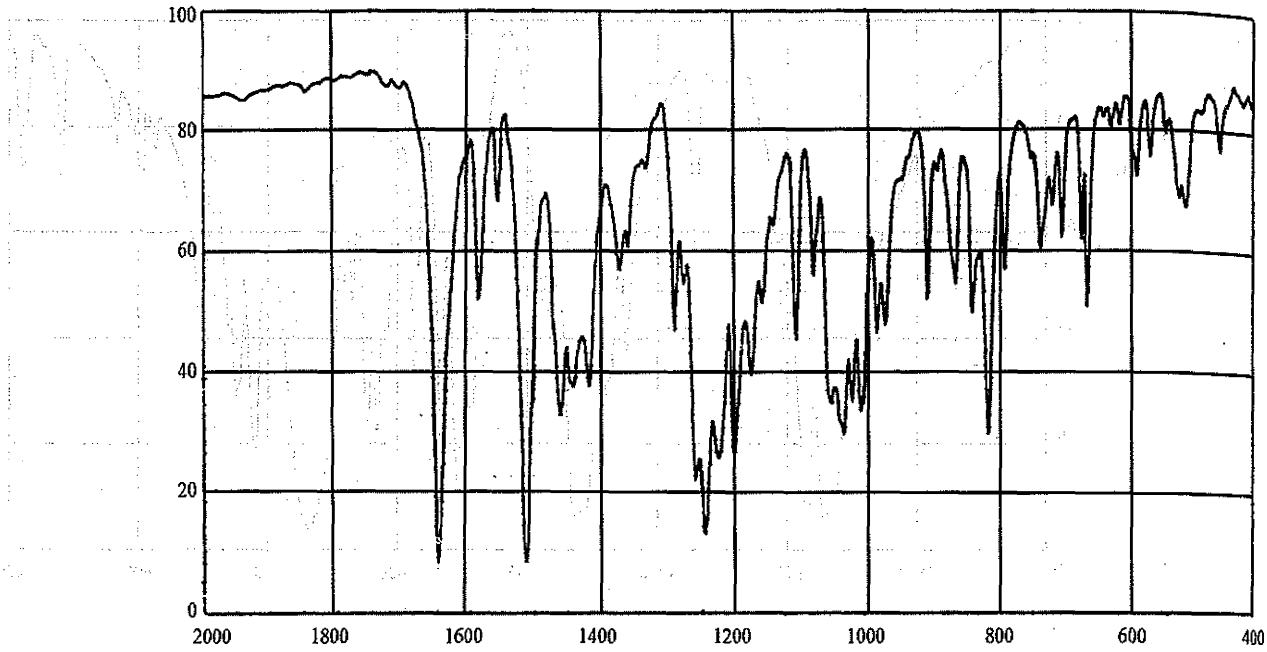
Isosorbide Dinitrate *FTIR* Phase: KBr disc



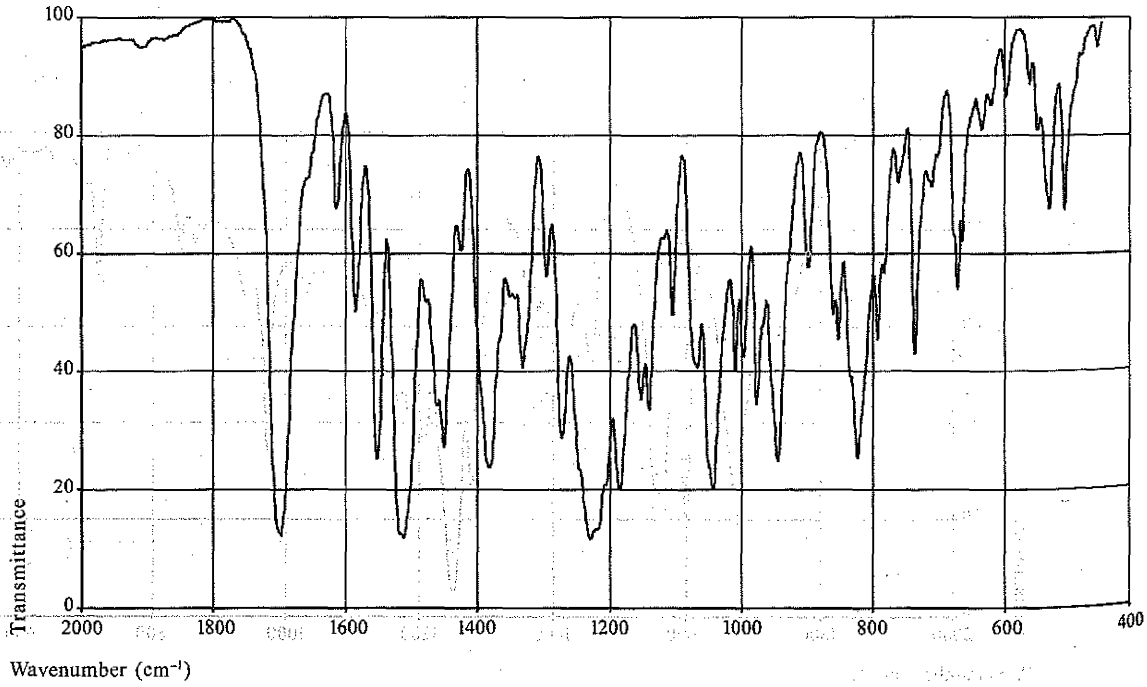
Isotretinoin *FTIR* Phase: KBr disc



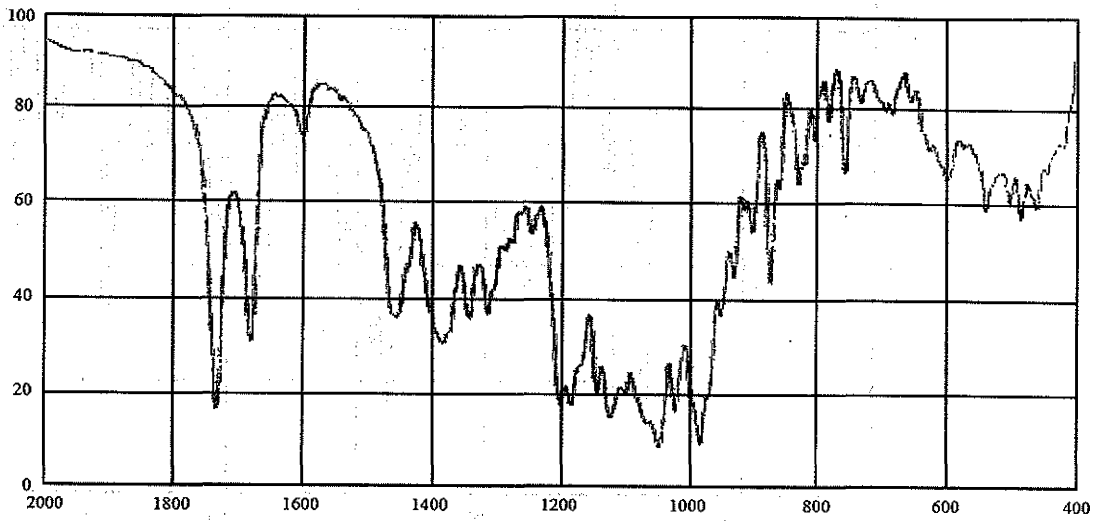
Isoxsuprine Hydrochloride *Dispersive IR* Phase: KCl disc



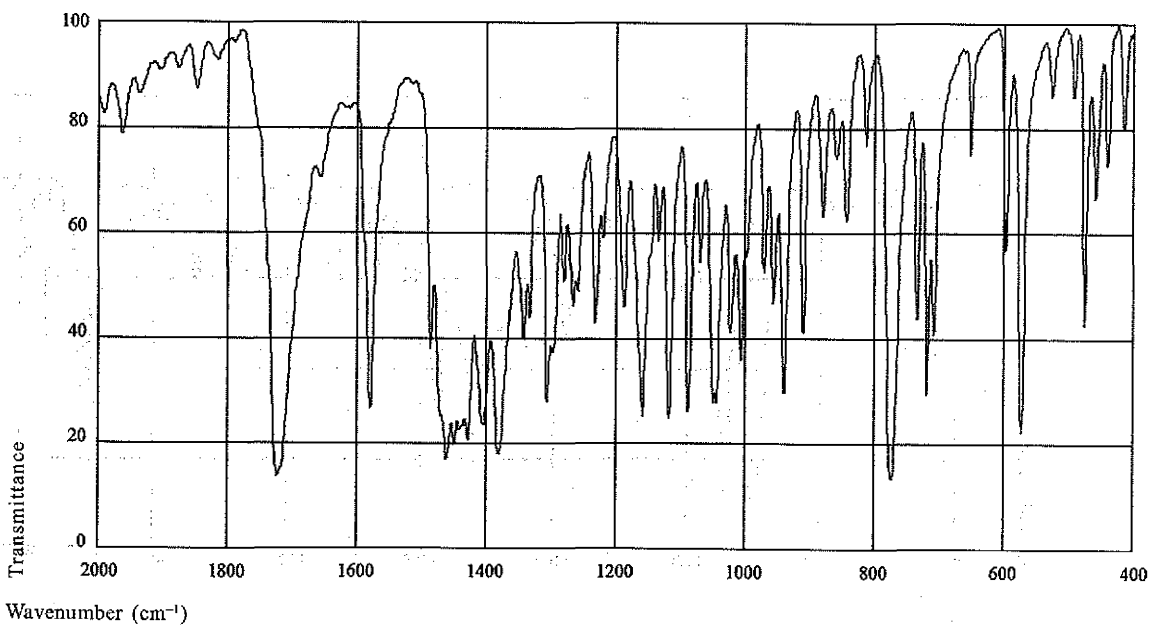
Itraconazole *FTIR* Phase: KBr disc



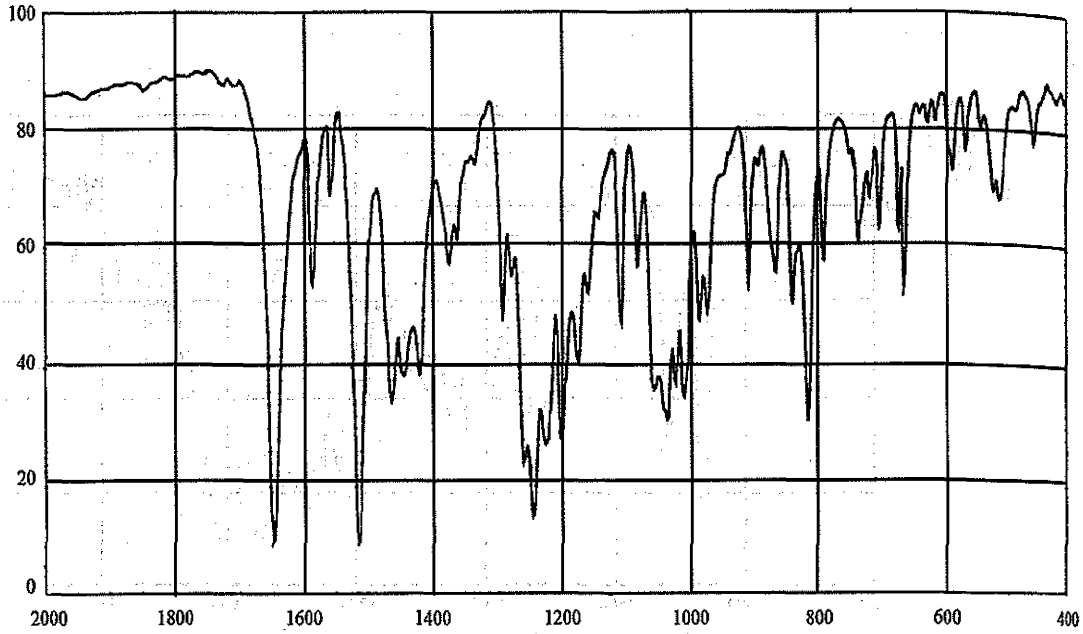
Ivermectin *FTIR* Phase: KBr disc



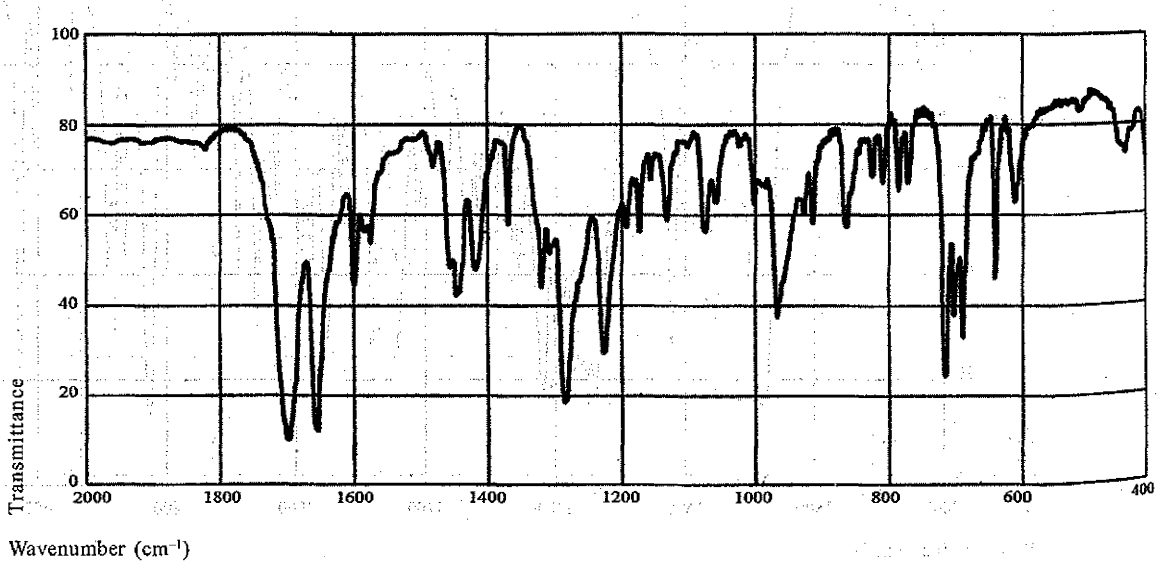
Ketamine Hydrochloride *FTIR* Phase: KCl disc



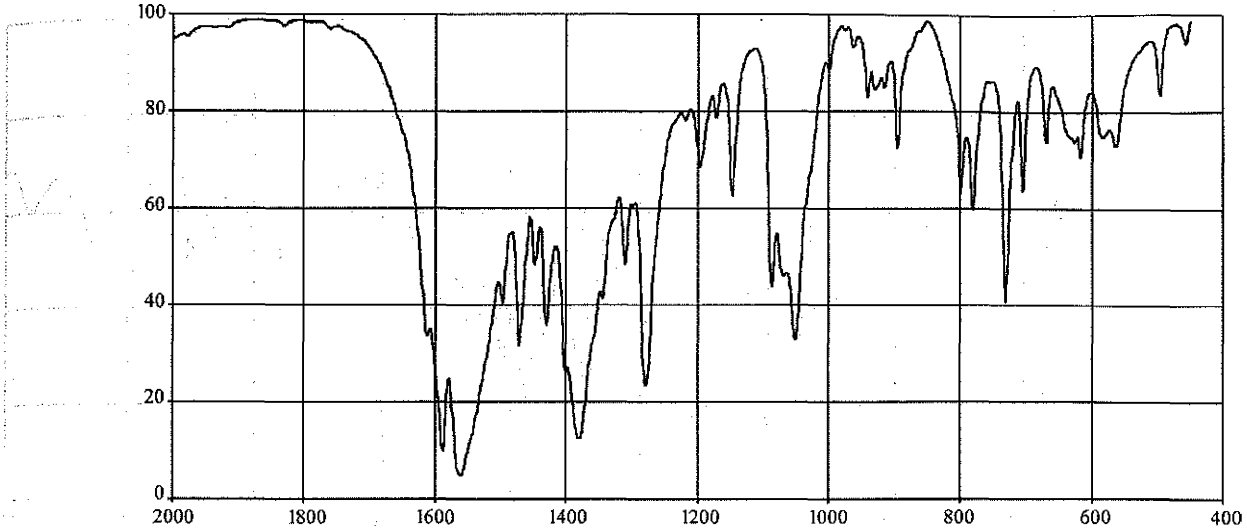
Ketoconazole *FTIR* Phase: KBr disc



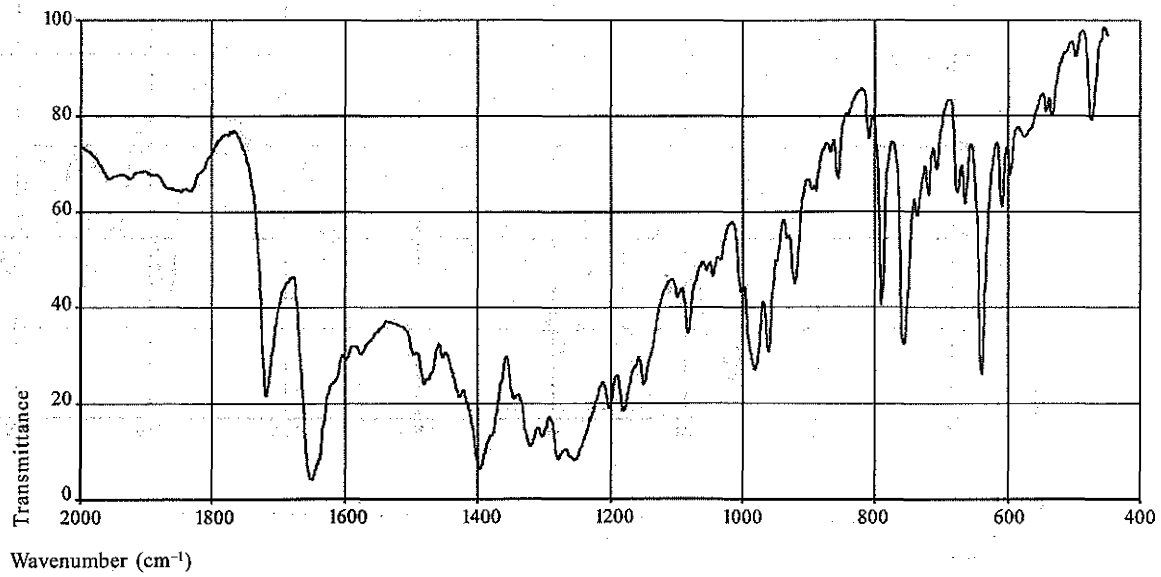
Ketoprofen *Dispersive IR* Phase: KBr disc



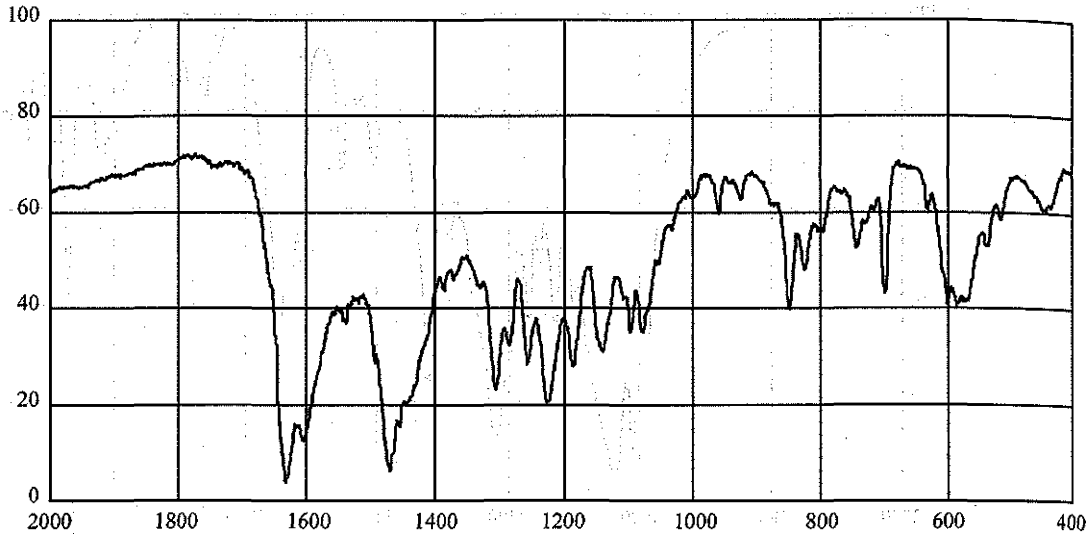
Ketorolac Tromethamine *Dispersive IR* Phase: KBr disc



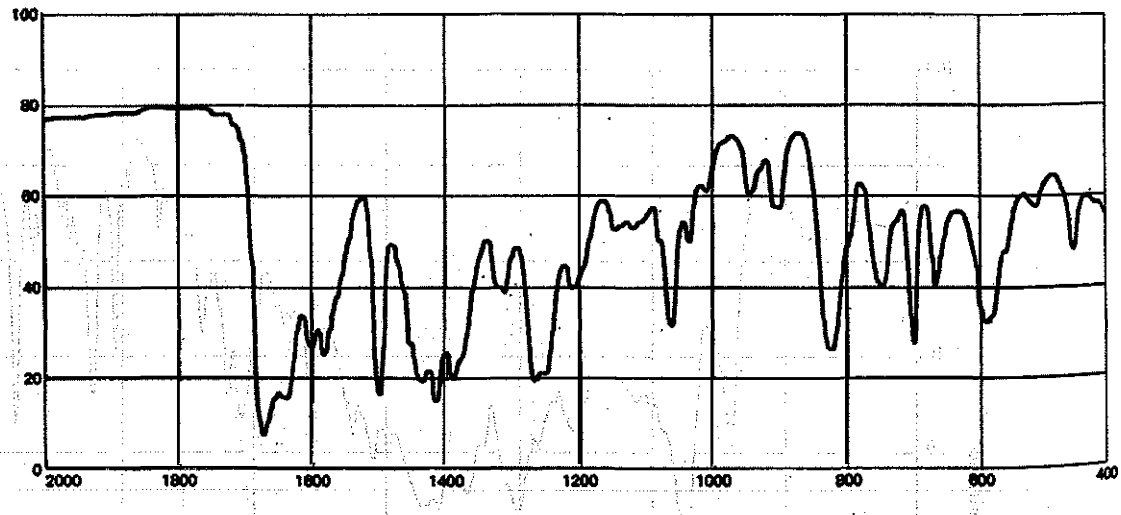
Ketotifen Hydrogen Fumarate *FTIR* Phase: KBr disc



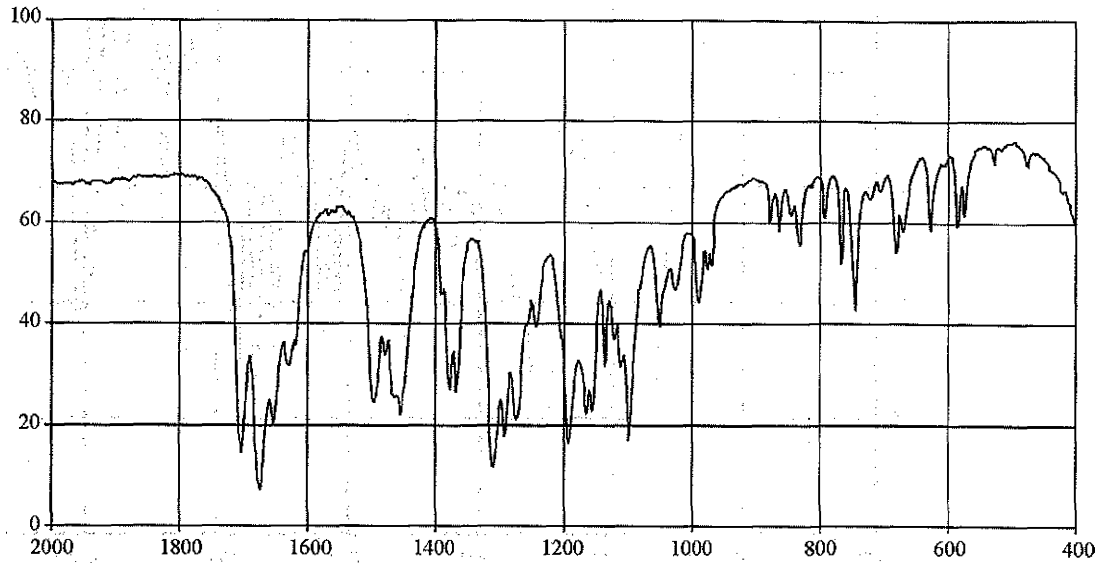
Labetalol *FTIR* Phase: KBr disc



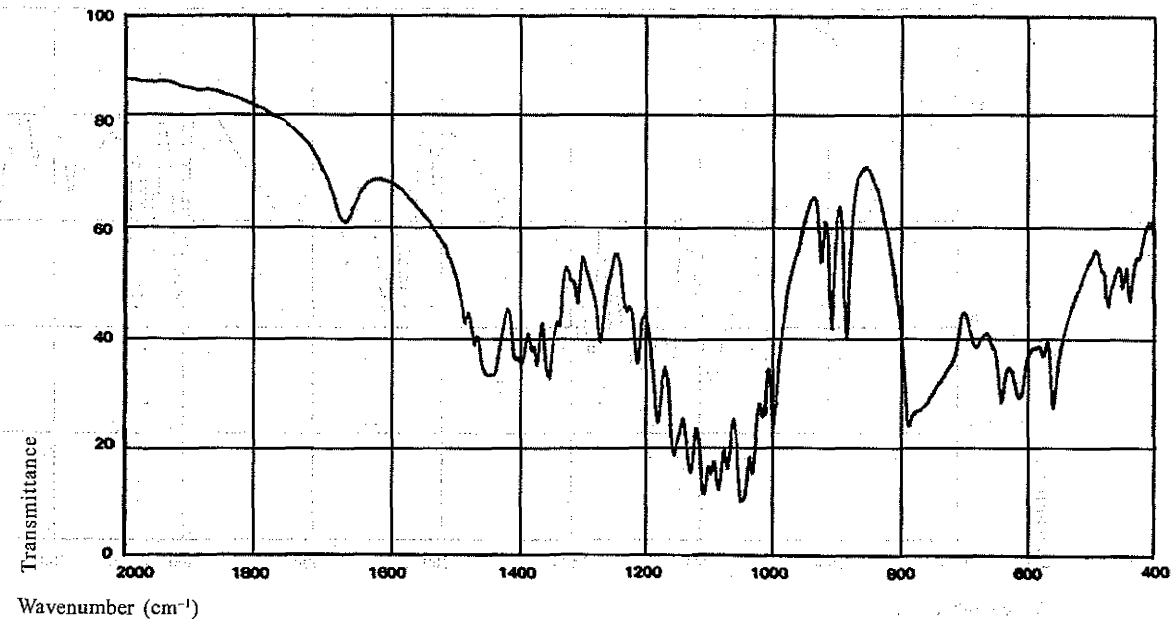
Labetalol Hydrochloride *Dispersive IR* Phase: KCl disc



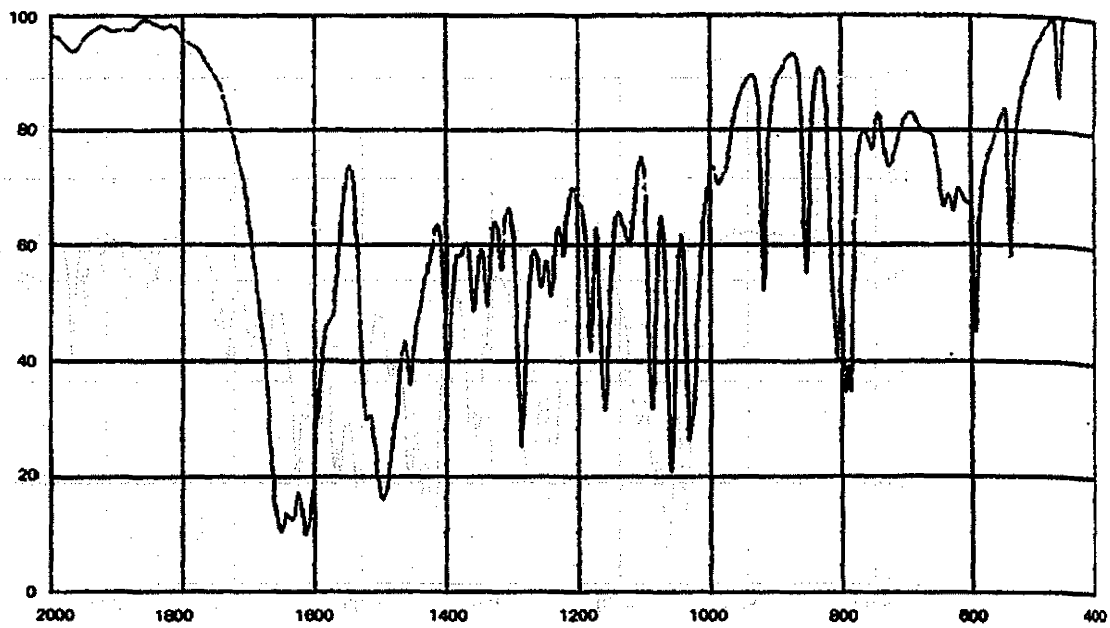
Lacidipine *FTIR* Phase: KBr disc



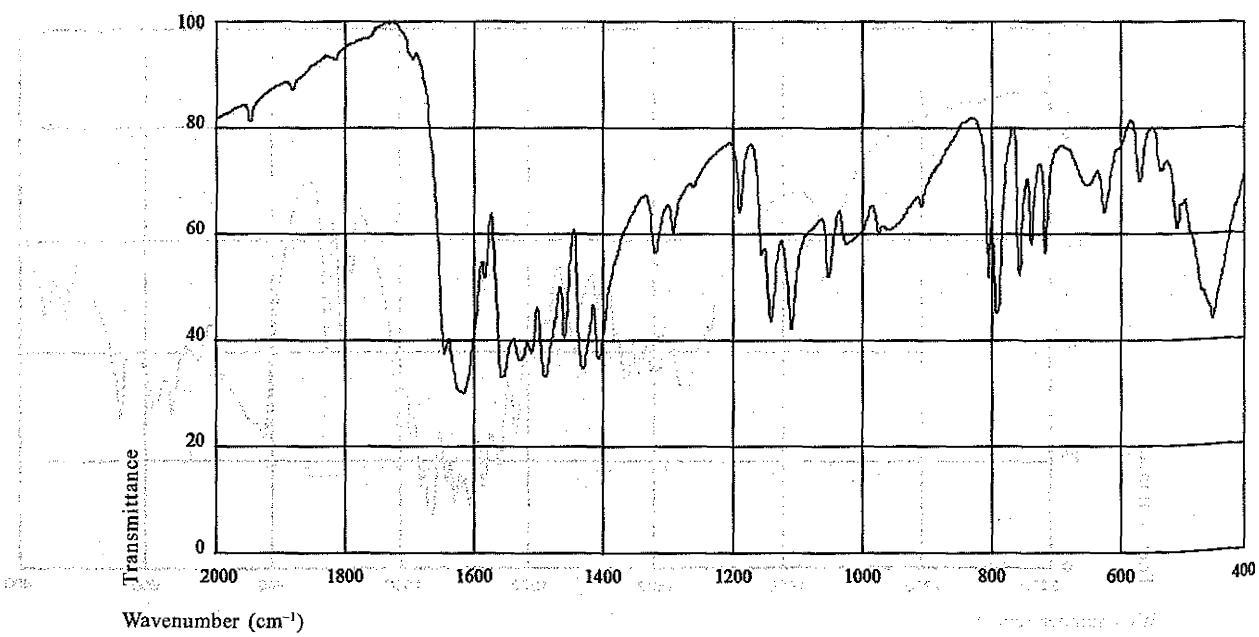
Lactose *FTIR* Phase: KBr disc



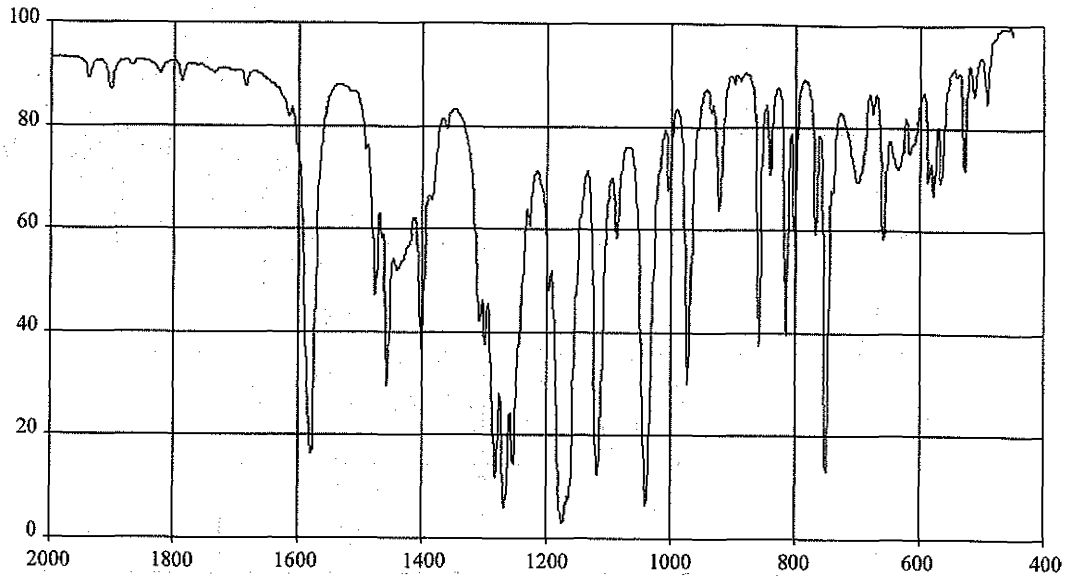
Lamivudine *FTIR* Phase: KBr disc



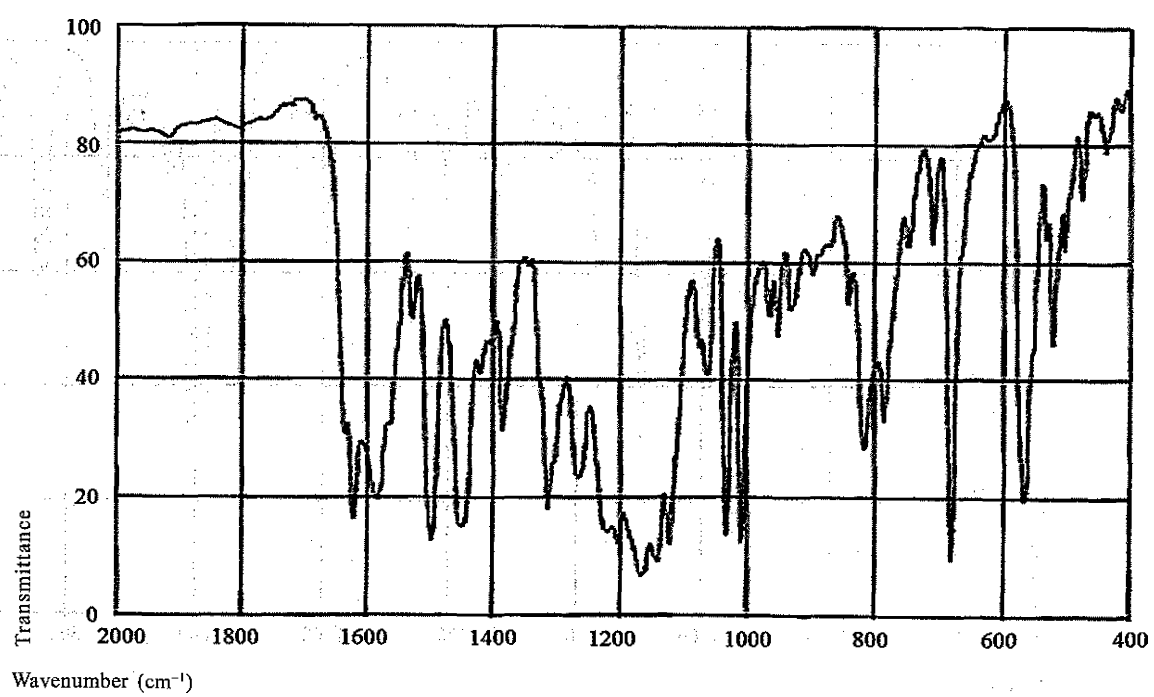
Lamotrigine *FTIR* Phase: KBr disc



Lansoprazole *FTIR* Phase: KBr disc



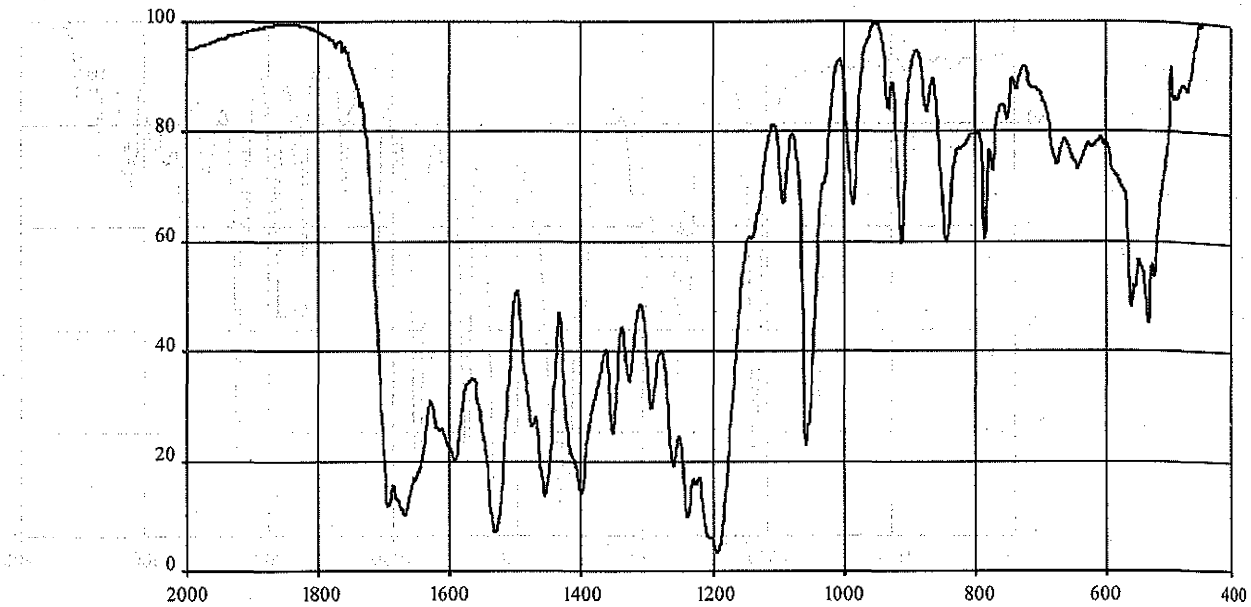
Lapatanib Ditosylate *FTIR* Phase: KBr disc



Lenvatinib Mesylate

FTIR

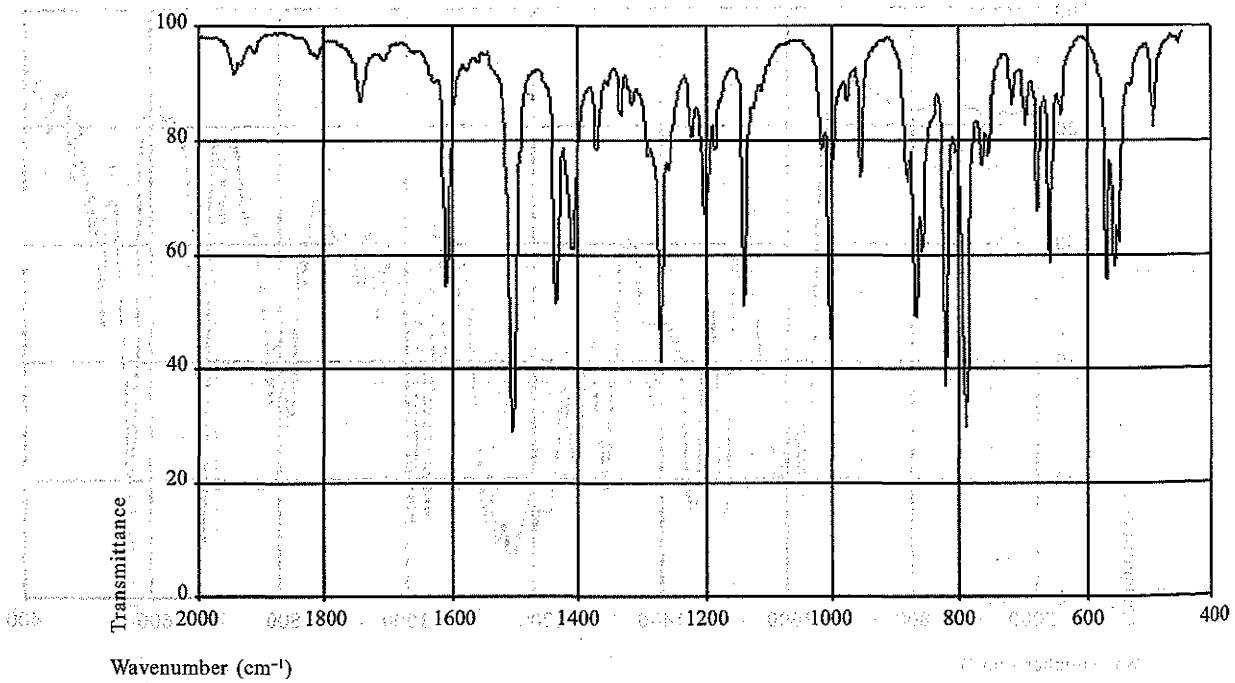
Phase: KBr disc



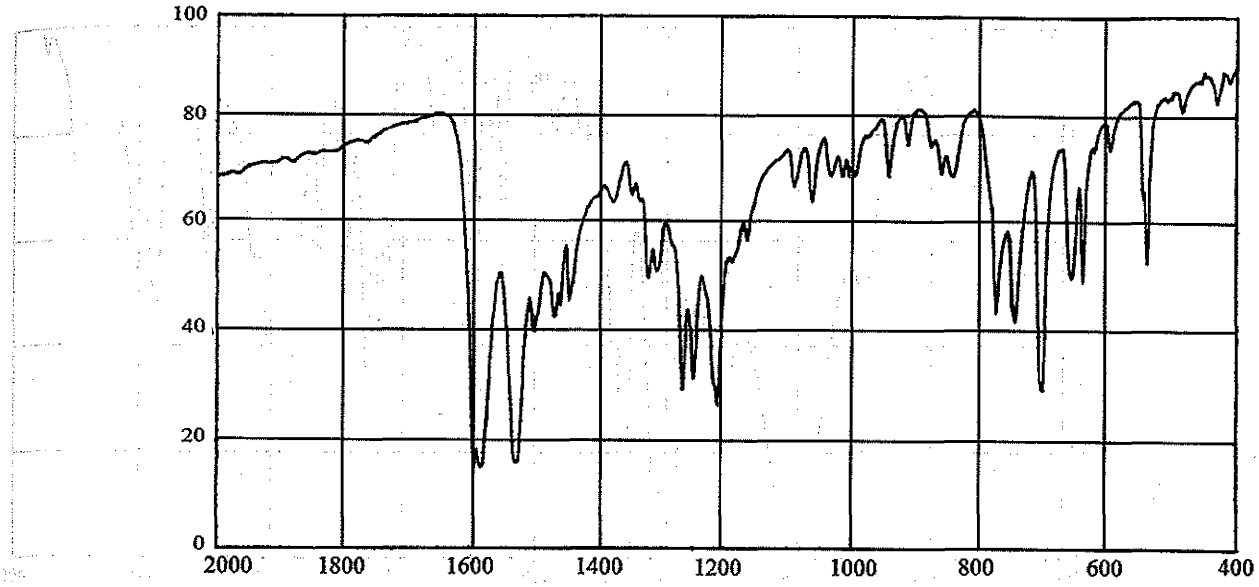
Letrozole

FTIR

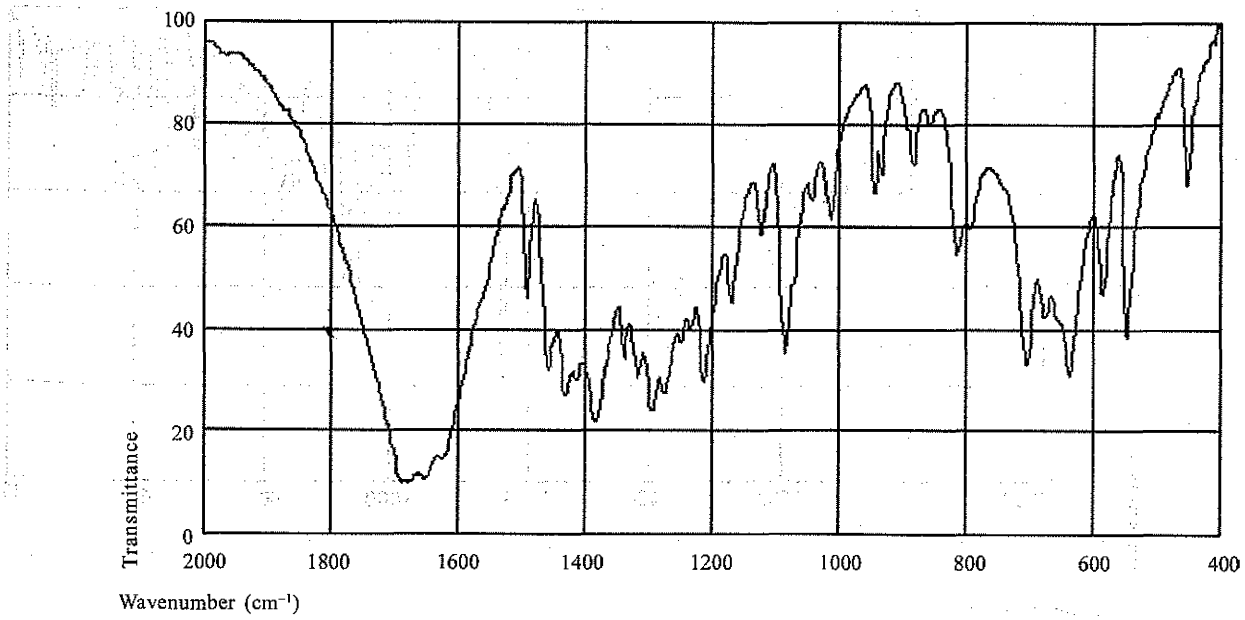
Phase: KBr disc



Levamisole Hydrochloride *FTIR* Phase: KBr disc



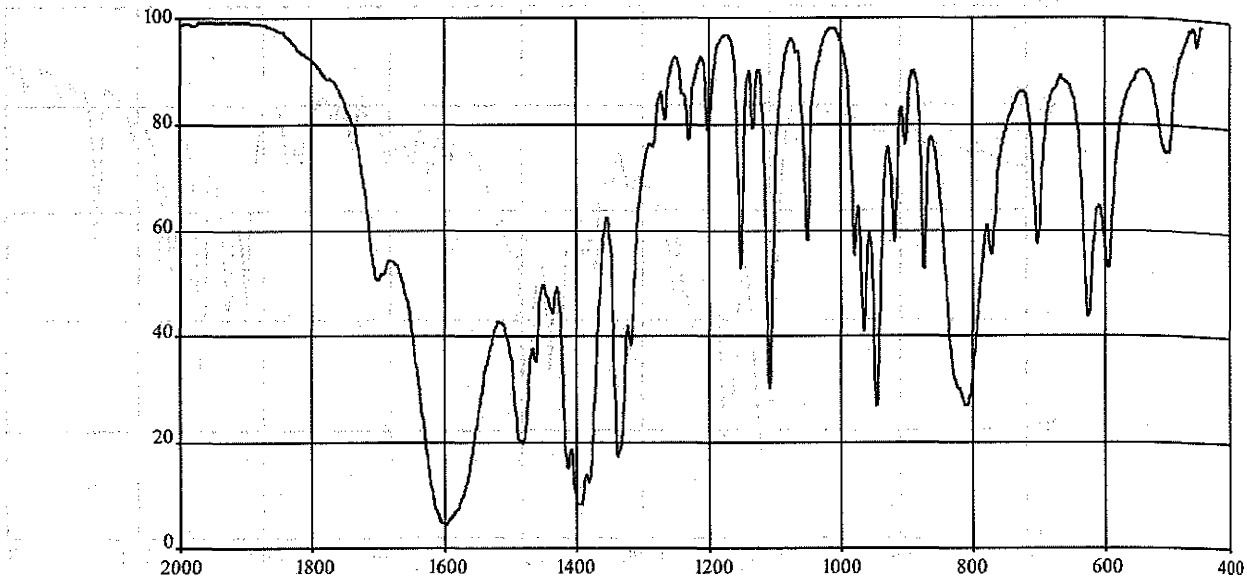
Levetiracetam *FTIR* Phase: KBr disc



Levocarnitine

FTIR

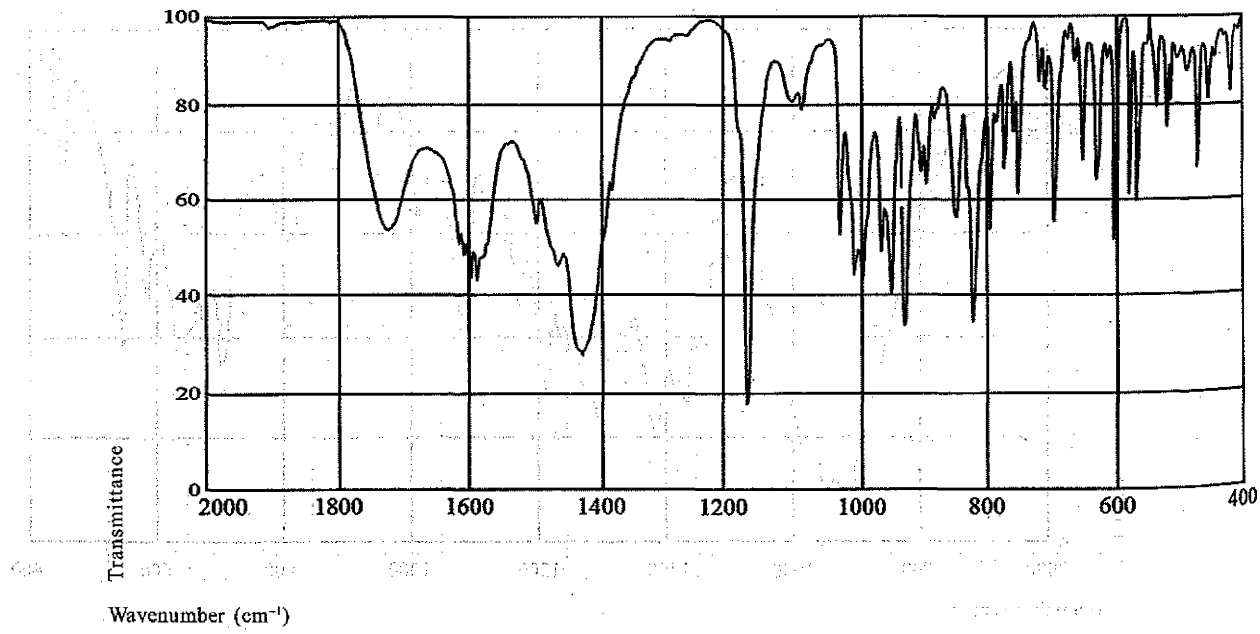
Phase: KBr disc



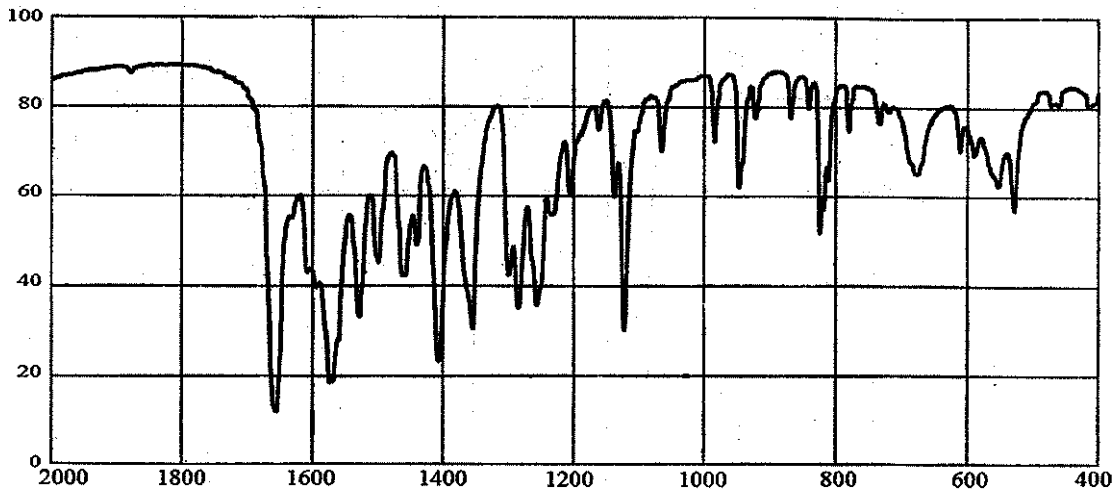
Levocetirizine Hydrochloride

FTIR

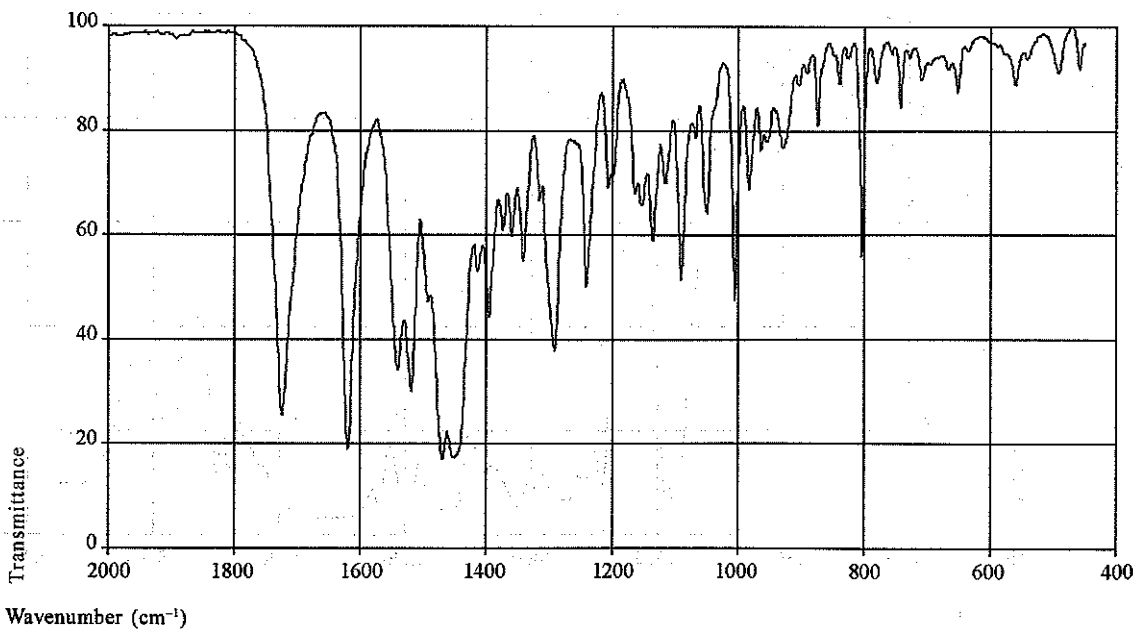
Phase: KBr disc



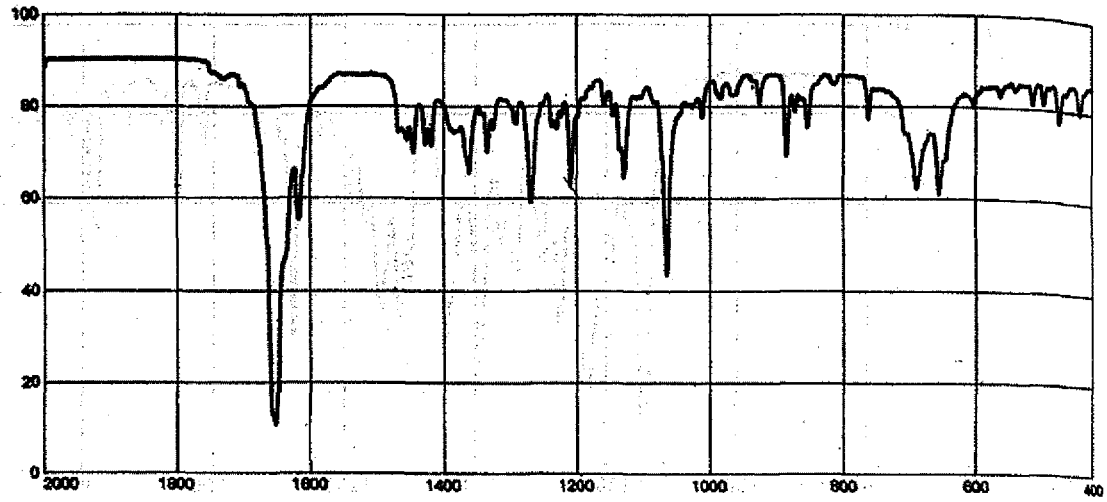
Levodopa *Dispersive IR* Phase: KBr disc



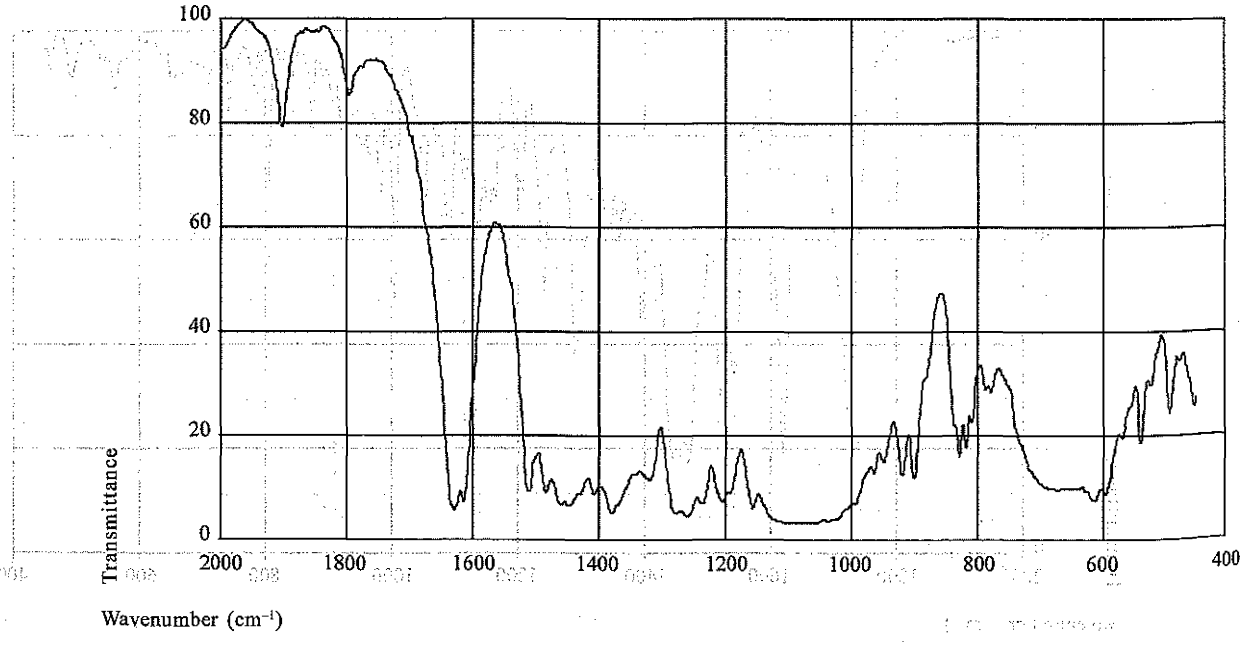
Levofloxacin Hemihydrate *FTIR* Phase: KBr disc



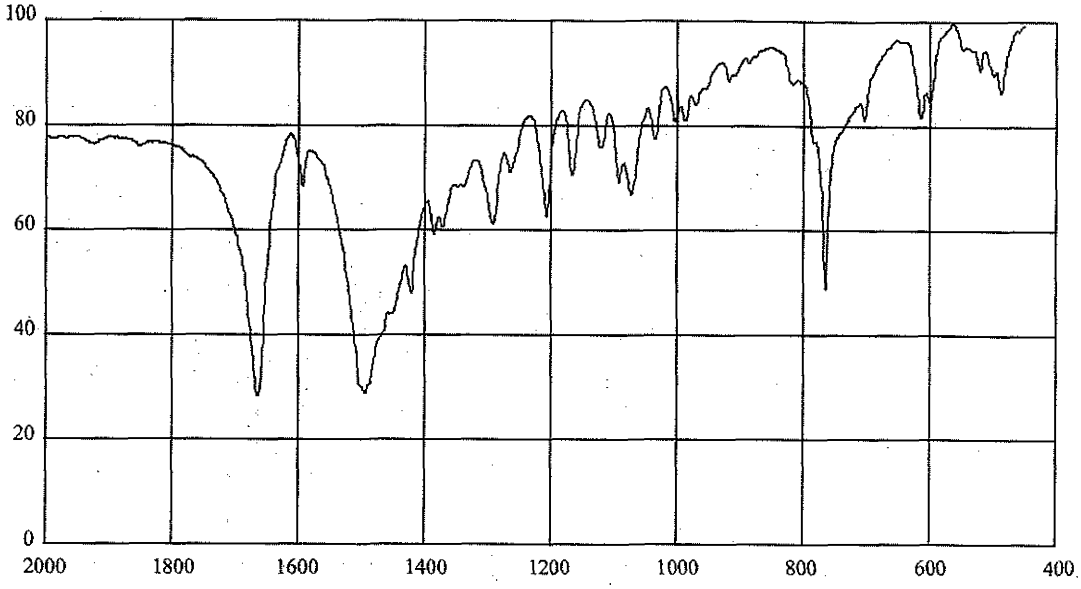
Levonorgestrel *Dispersive IR* Phase: KBr disc



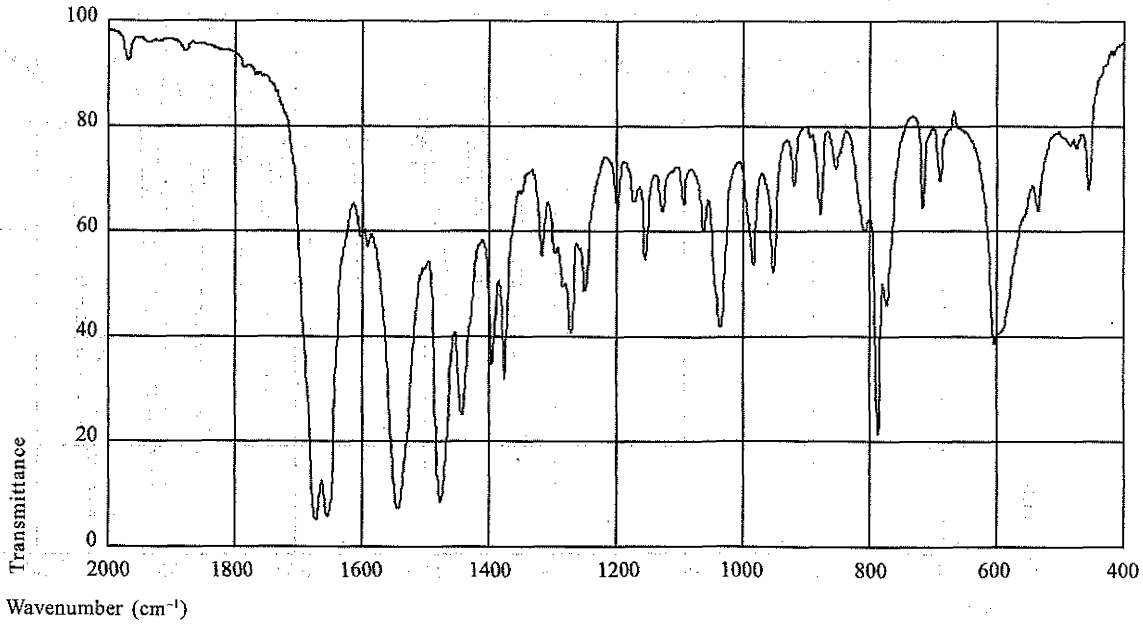
Levosalbutamol Sulphate *FTIR* Phase: KBr disc



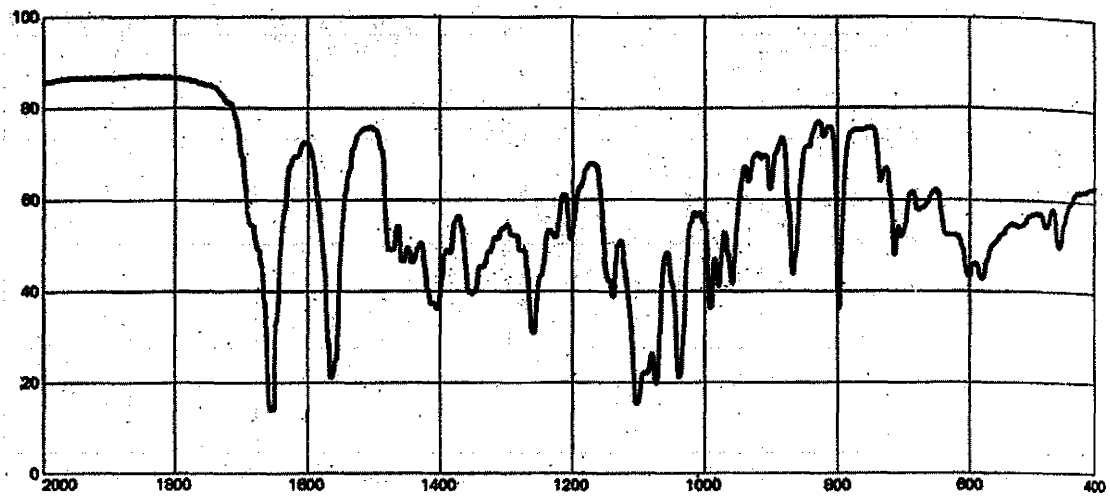
Lignocaine *FTIR* Phase: KBr disc



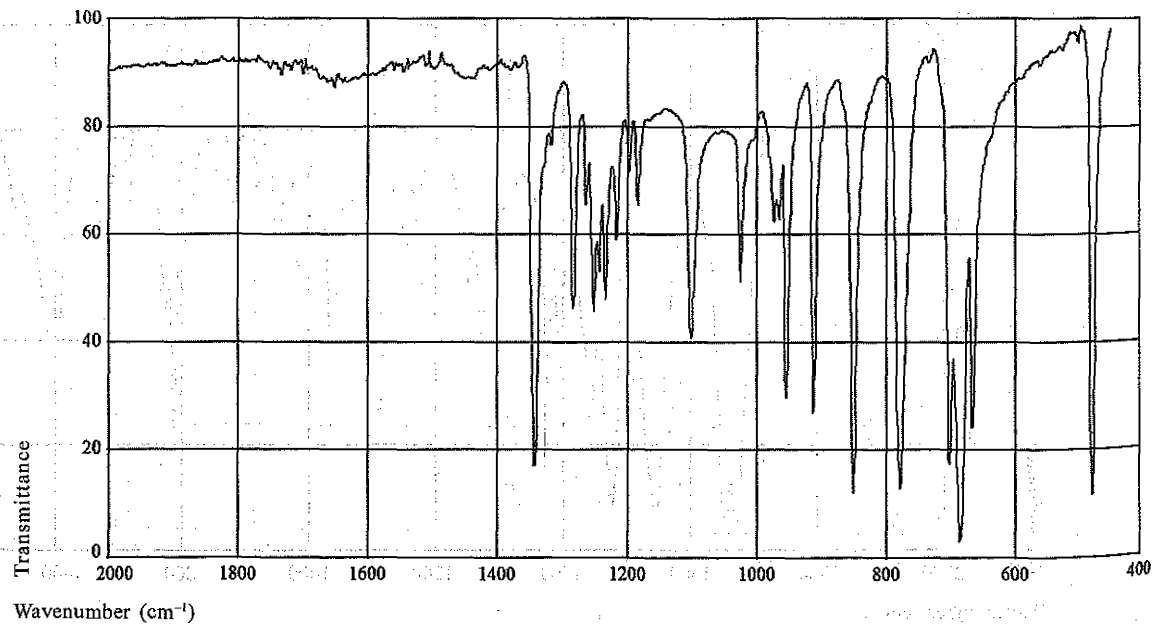
Lignocaine Hydrochloride *FTIR* Phase: KBr disc



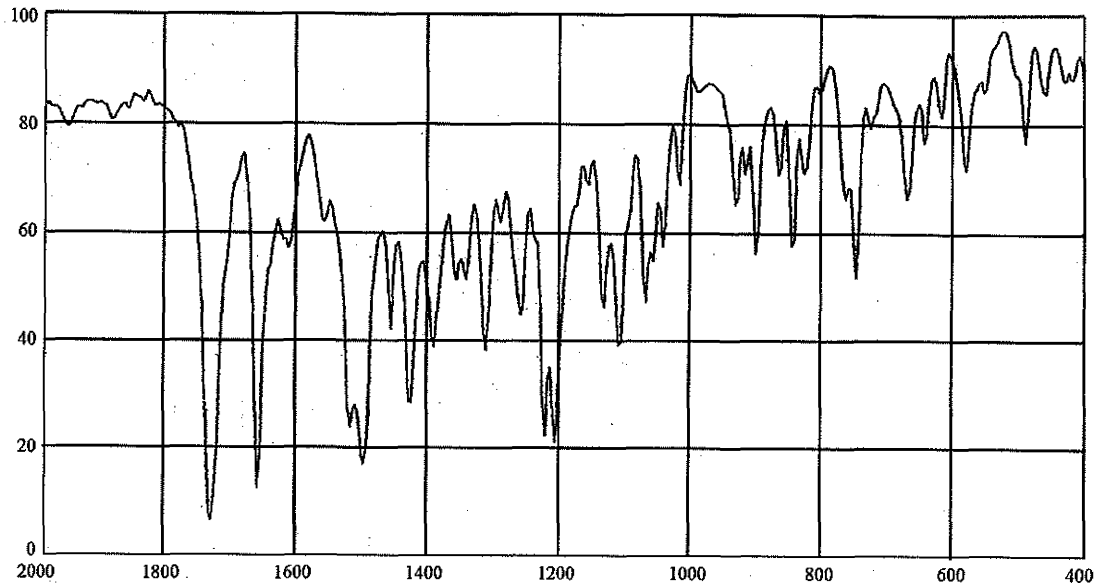
Lincomycin Hydrochloride *Dispersive IR* Phase: KCl disc



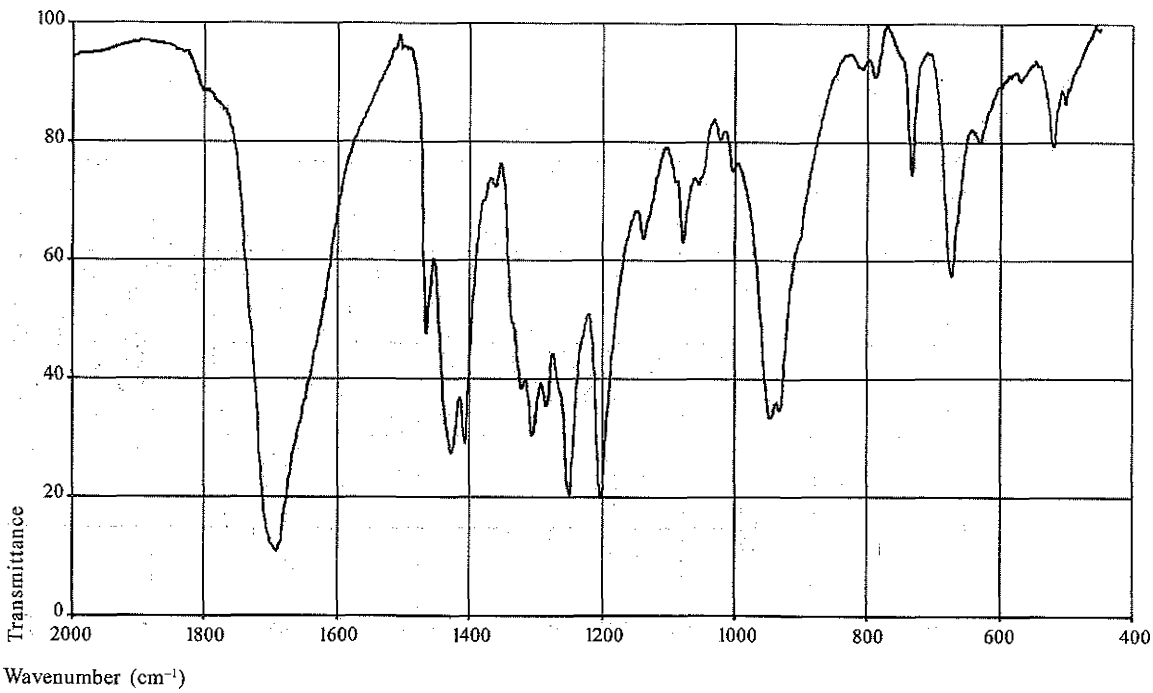
Lindane *FTIR* Phase: KBr disc



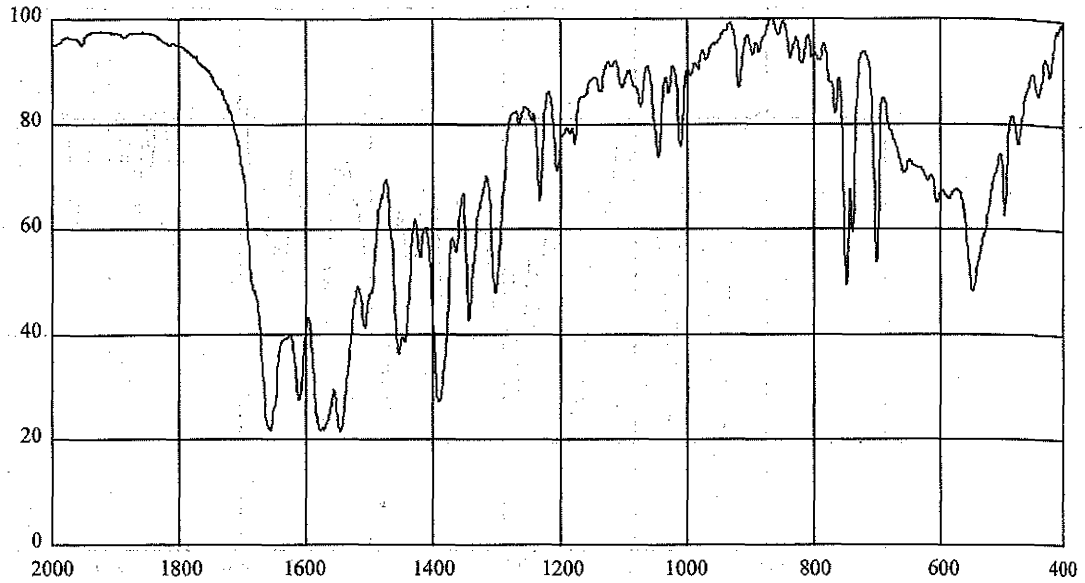
Linezolid *FTIR* Phase: KBr disc



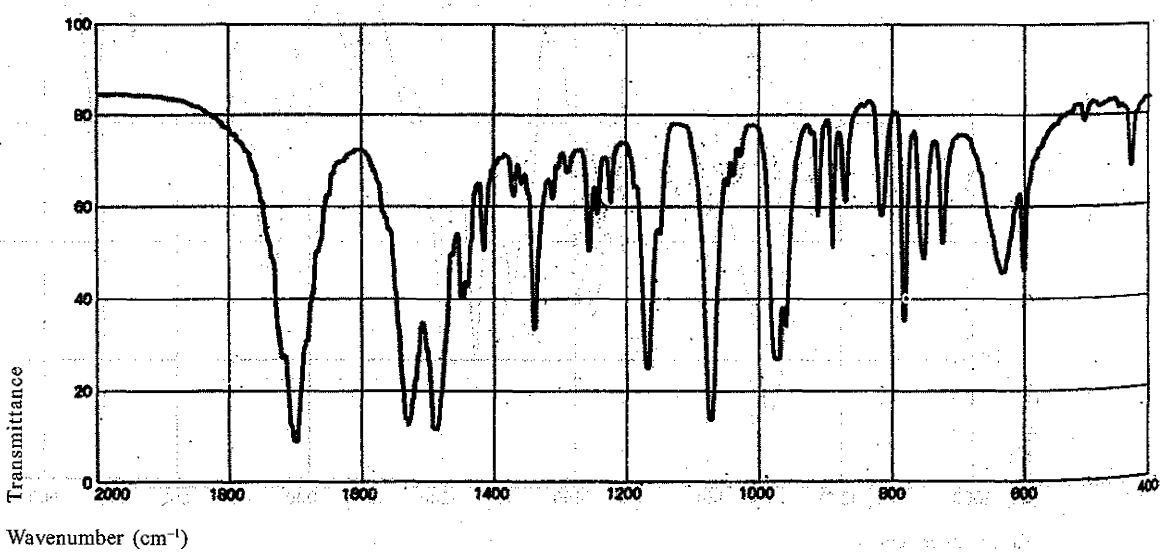
Alpha Lipoic Acid *FTIR* Phase: KBr disc



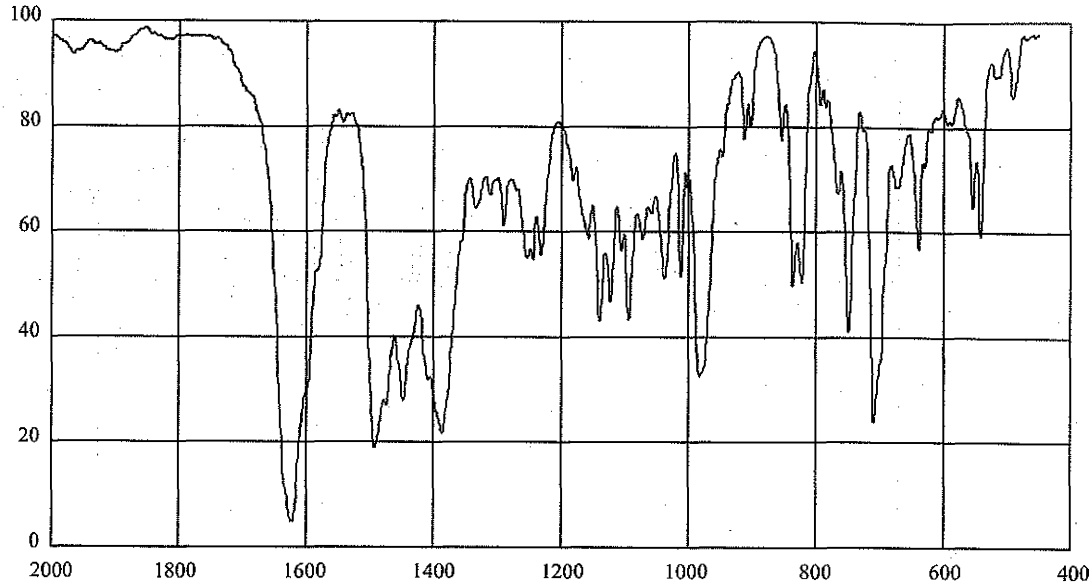
Lisinopril *FTIR* Phase: Liquid paraffin mull



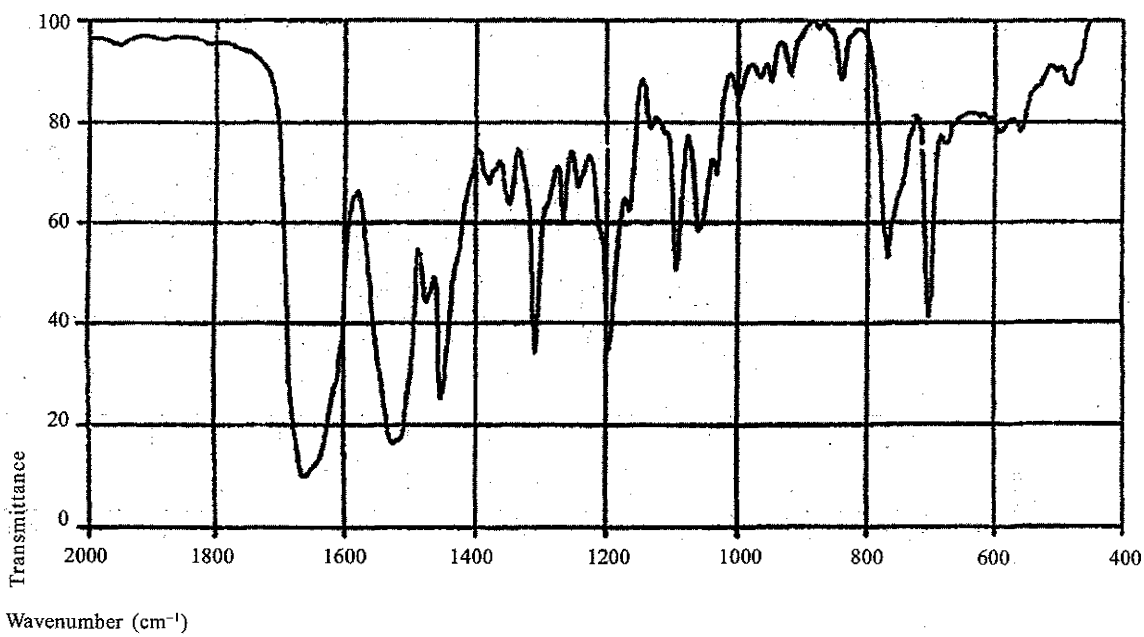
Lomustine *Dispersive IR* Phase: KBr disc



Loperamide Hydrochloride *FTIR* Phase: KBr disc



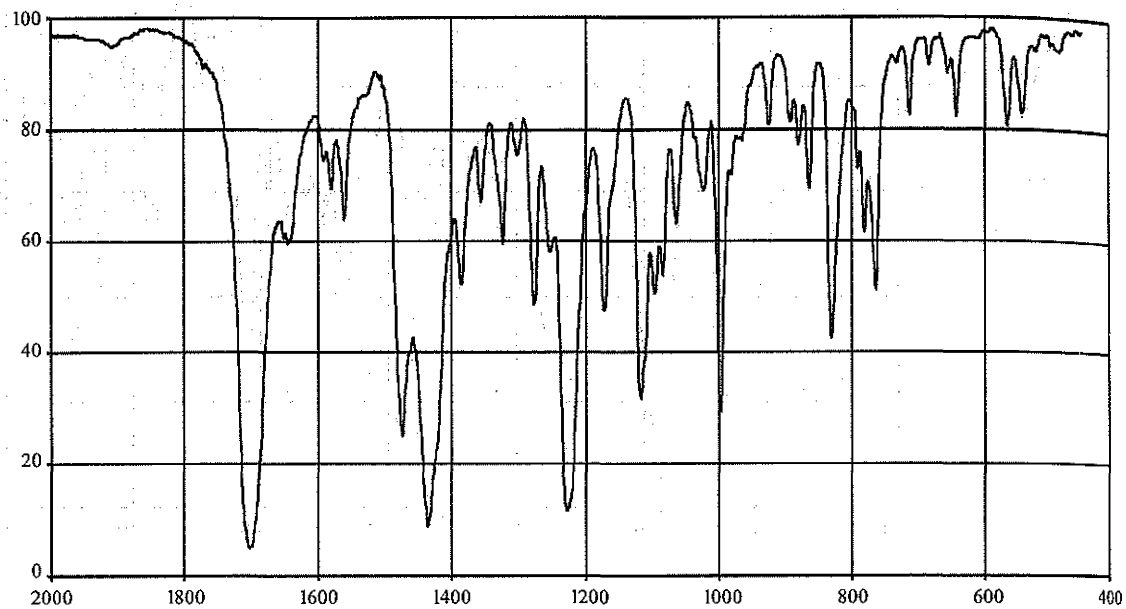
Lopinavir *FTIR* Phase: KBr disc



Loratadine

FTIR

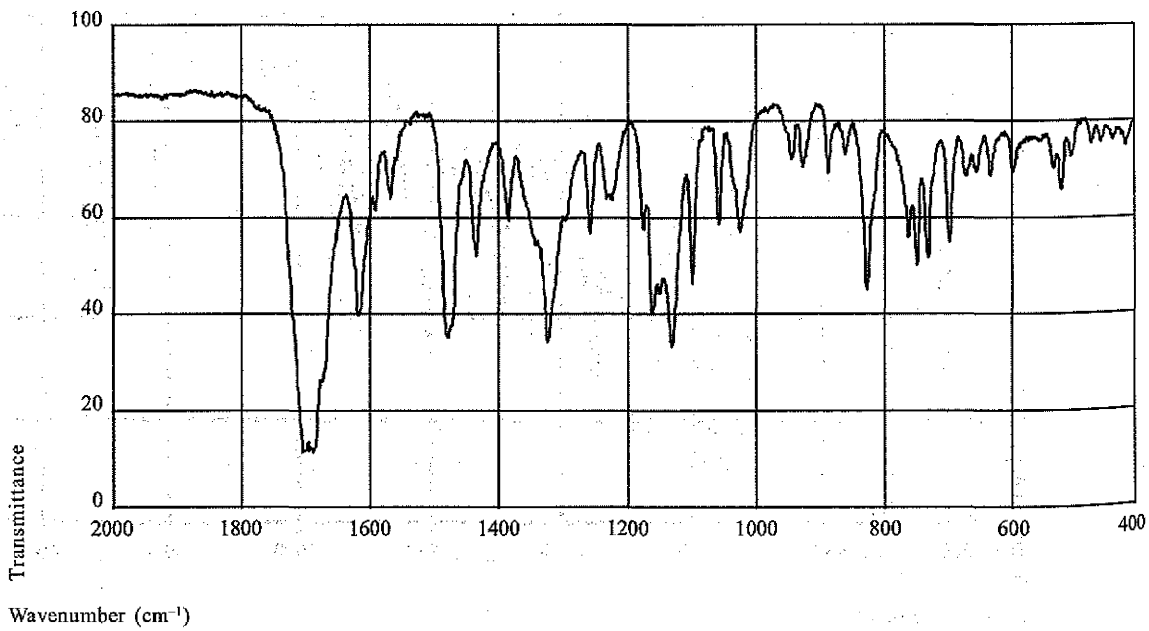
Phase: KBr disc



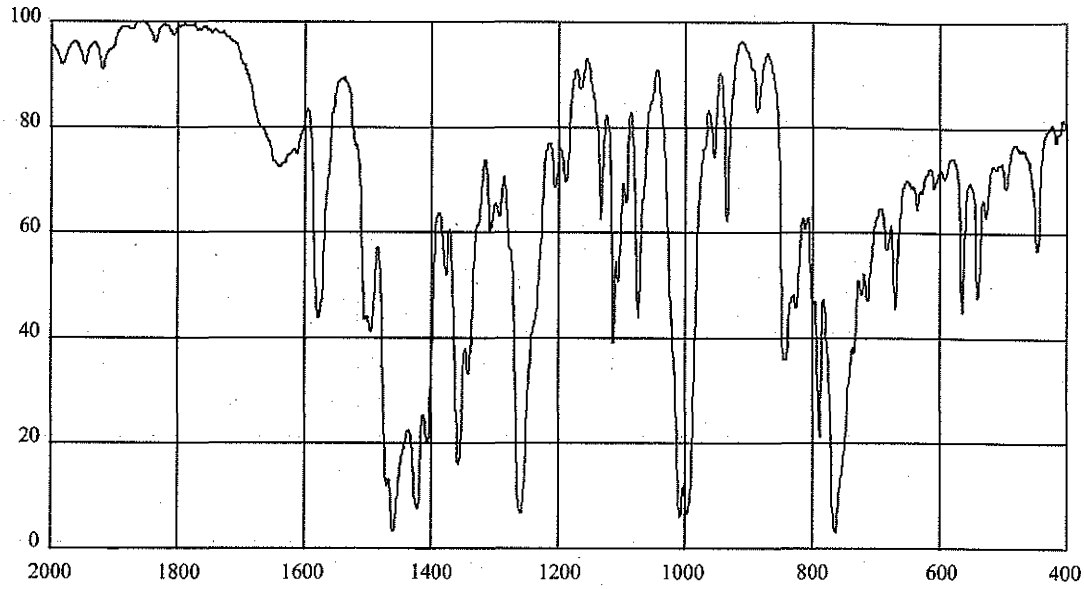
Lorazepam

FTIR

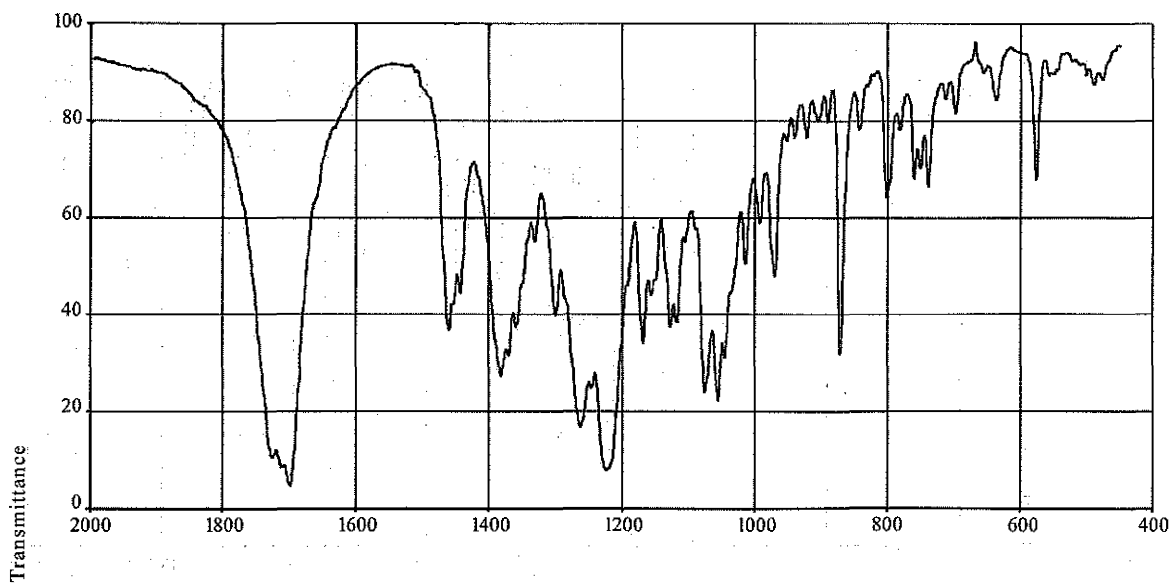
Phase: KBr disc



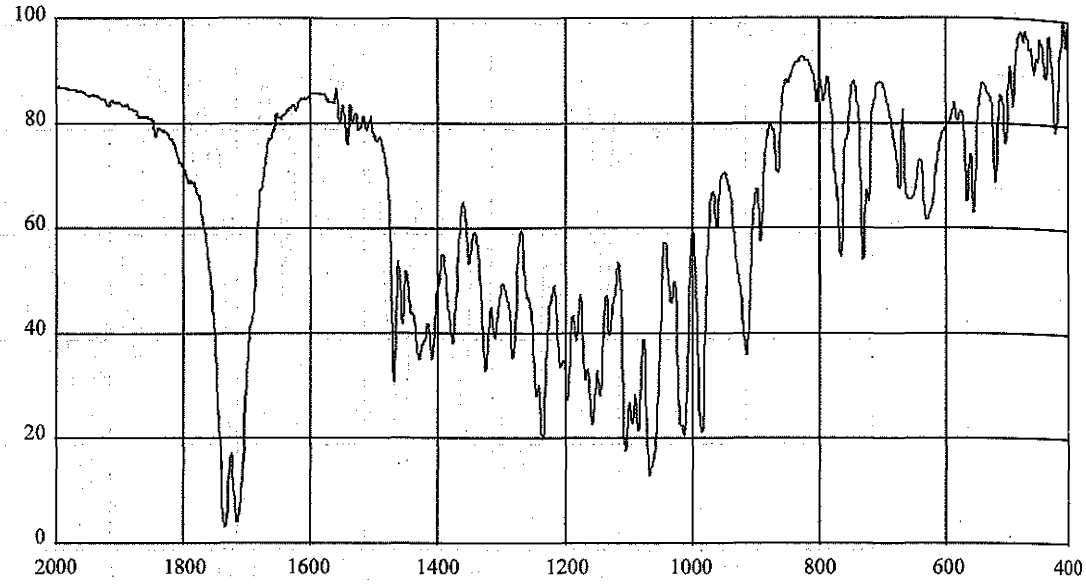
Losartan Potassium *FTIR* Phase: Liquid paraffin mull



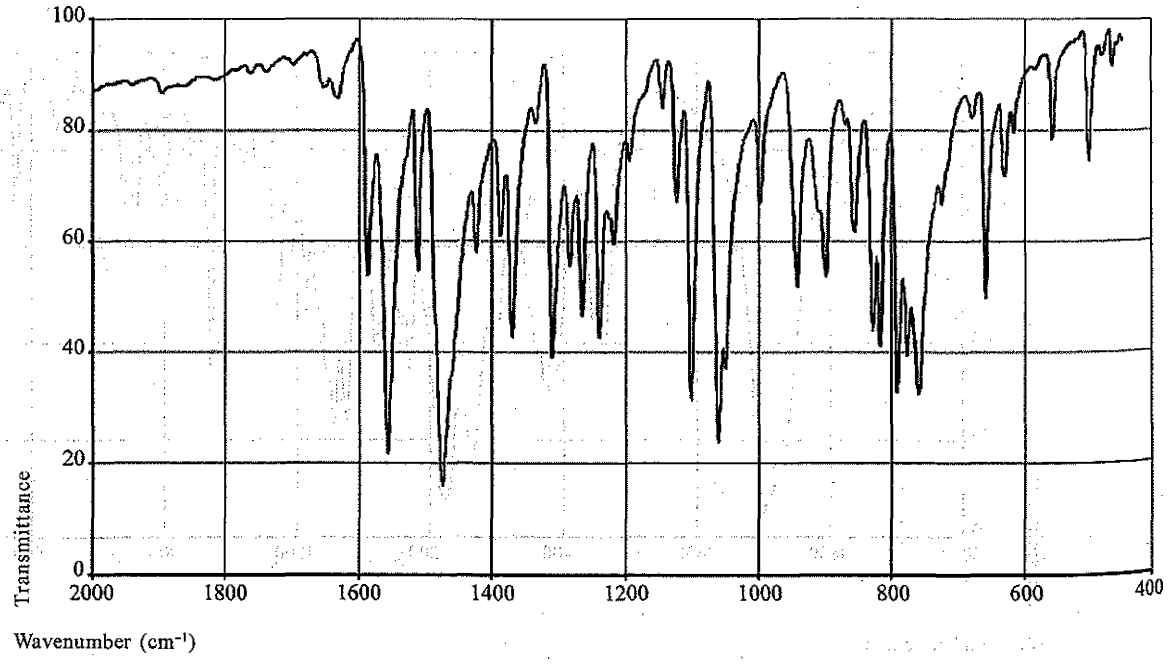
Lovastatin *FTIR* Phase: KBr disc



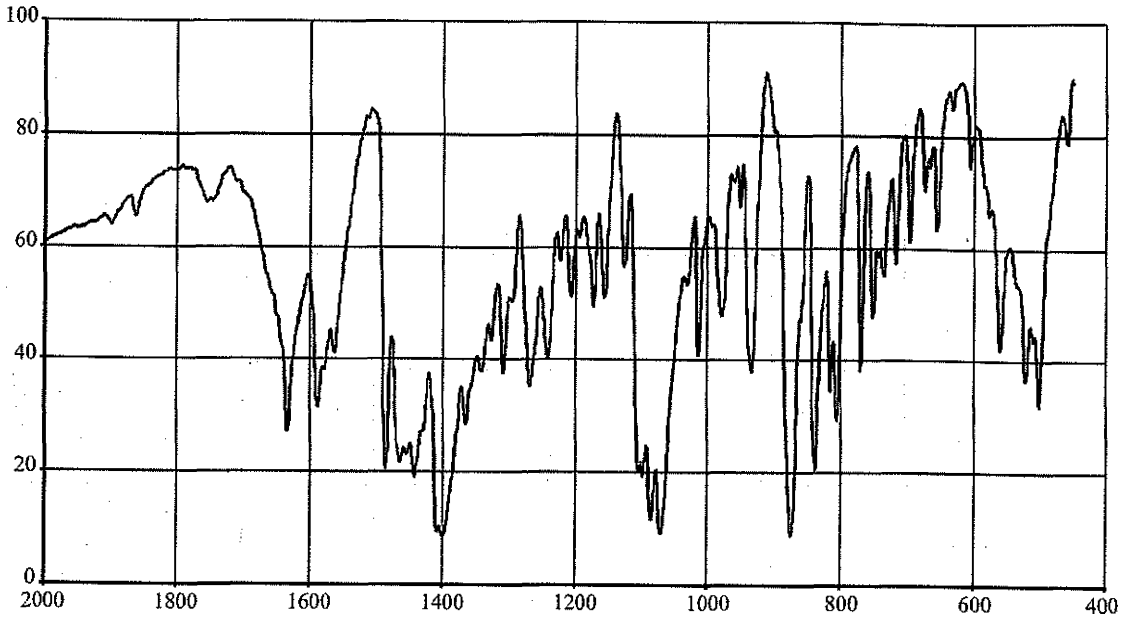
Lubiprostone *FTIR* Phase: KBr disc



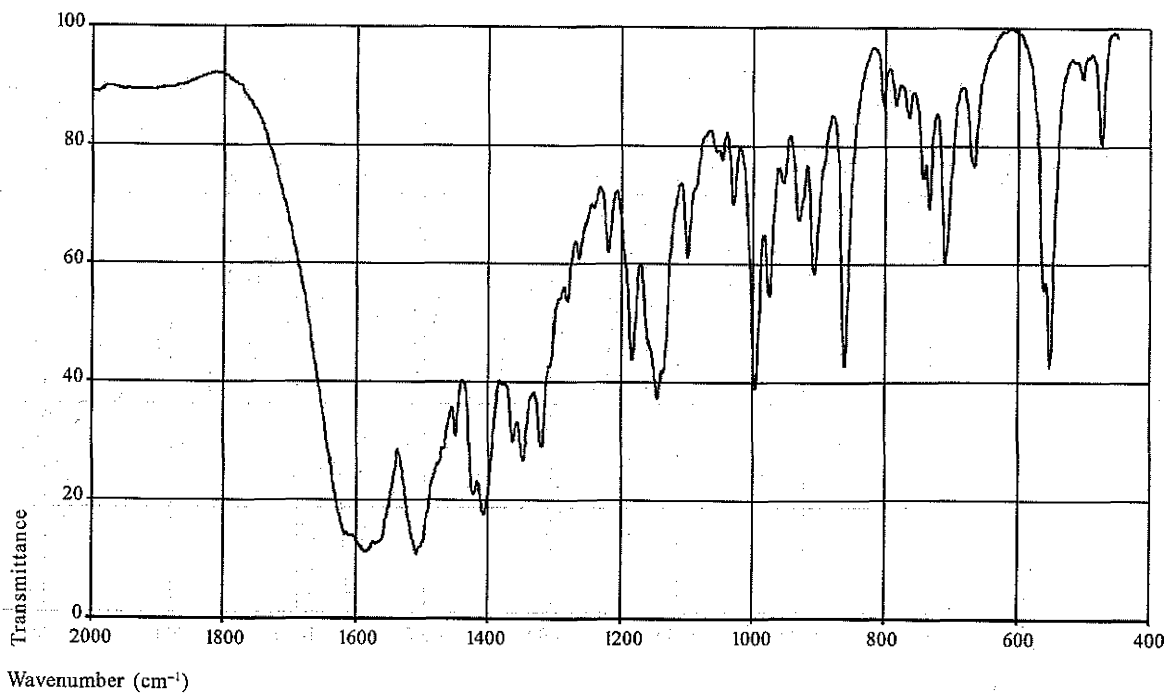
Luliconazole *FTIR* Phase: KBr disc



Lumefantrine *FTIR* Phase: KBr disc



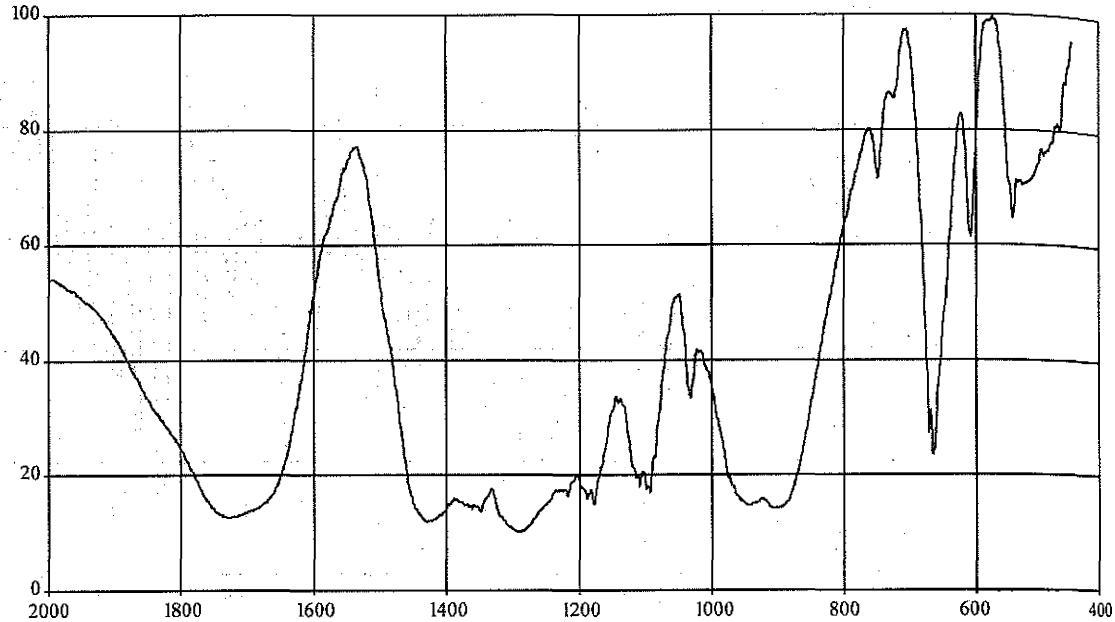
Lysine Hydrochloride *FTIR* Phase: KBr disc



Malic Acid

FTIR

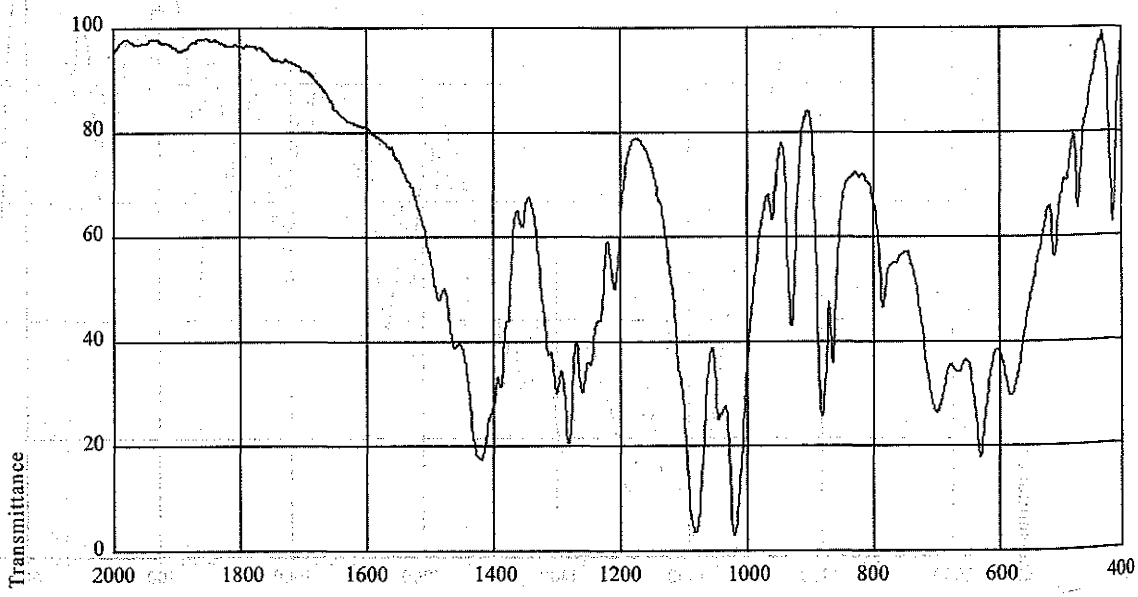
Phase: KBr disc



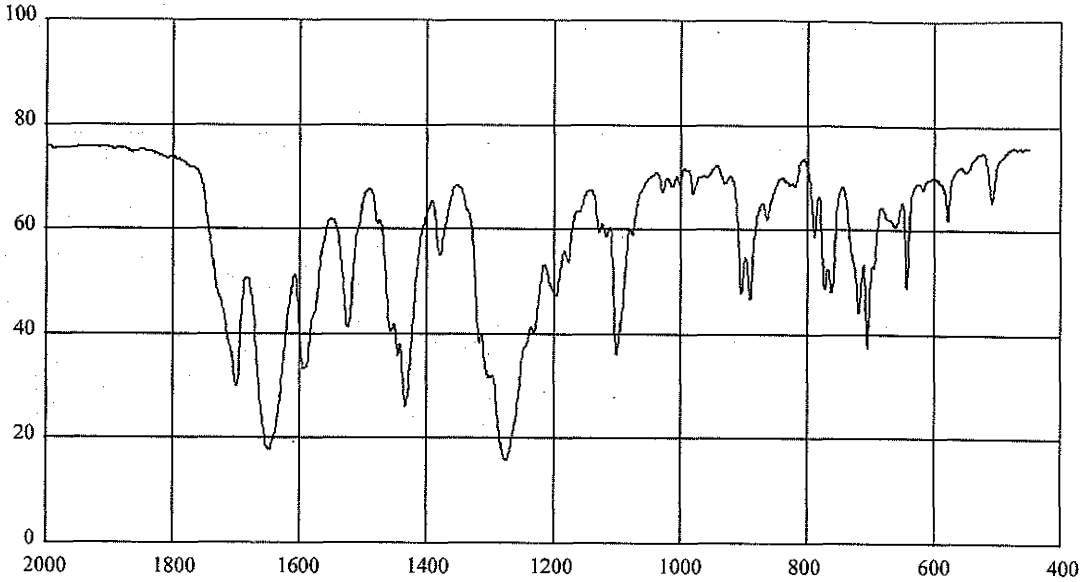
Mannitol

FTIR

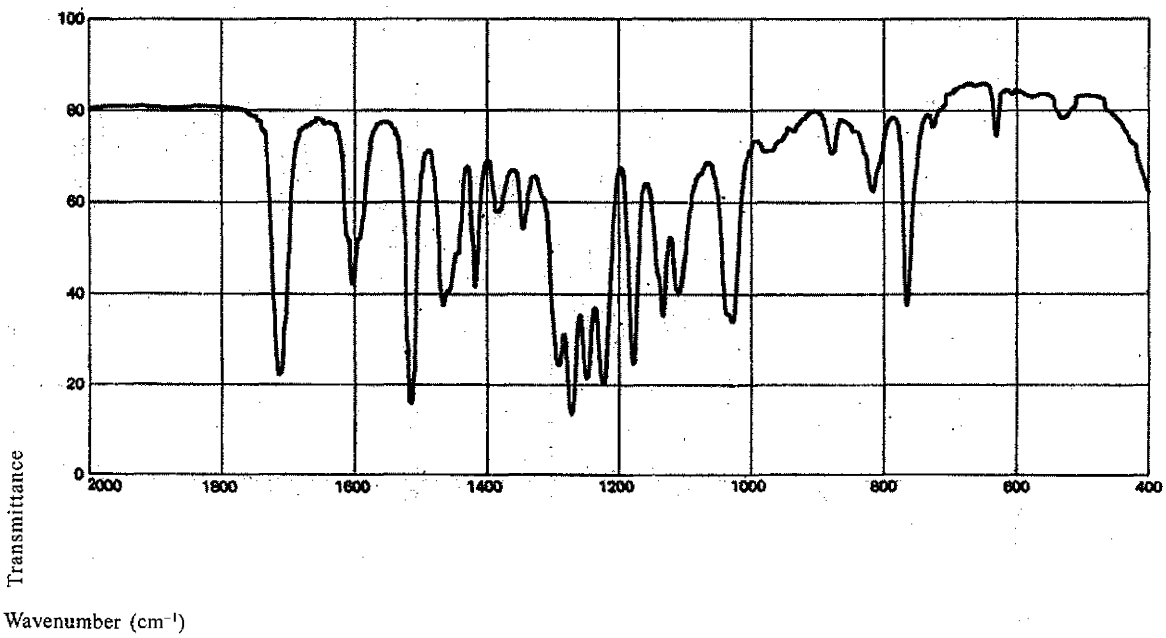
Phase: KBr disc



Mebendazole *FTIR* Phase: KBr disc



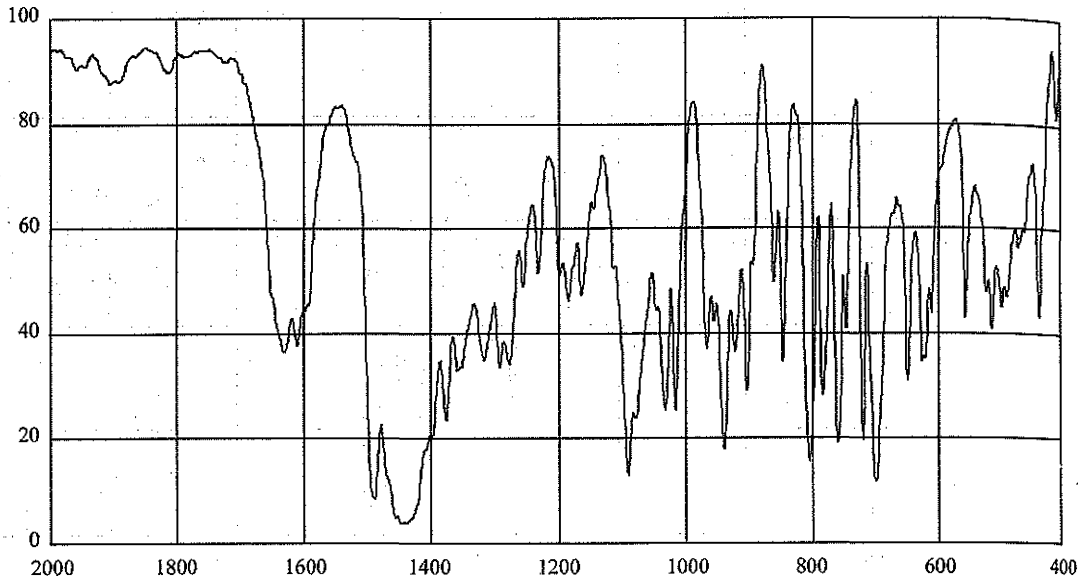
Mebeverine Hydrochloride *Dispersive IR* Phase: KCl disc



Meclizine Hydrochloride

FTIR

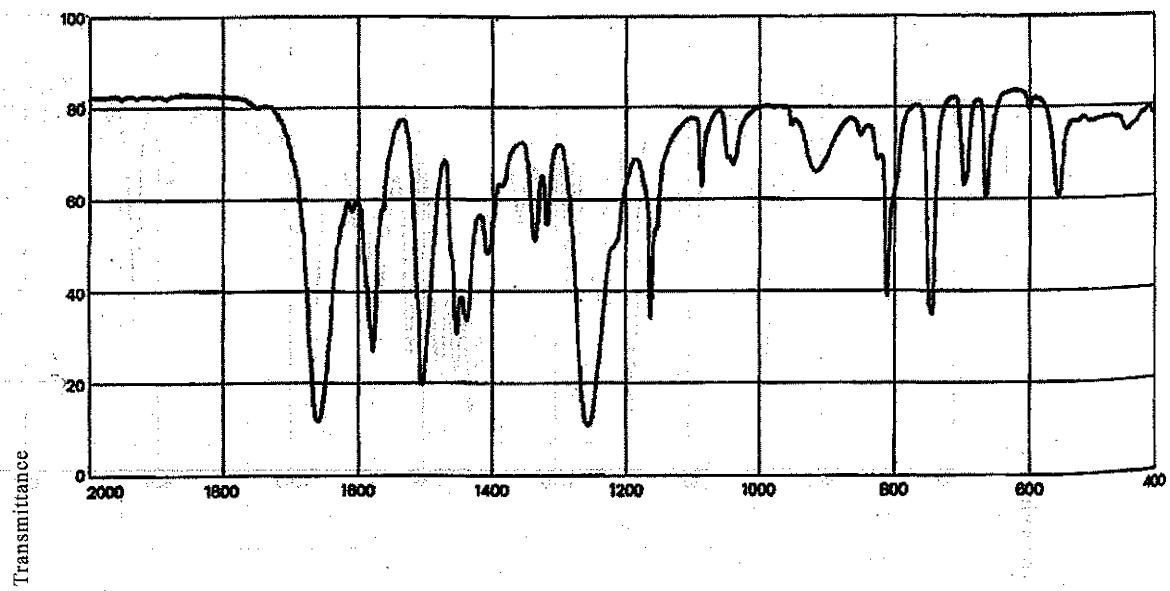
Phase: KCl disc



Meclofenamic Acid

Dispersive IR

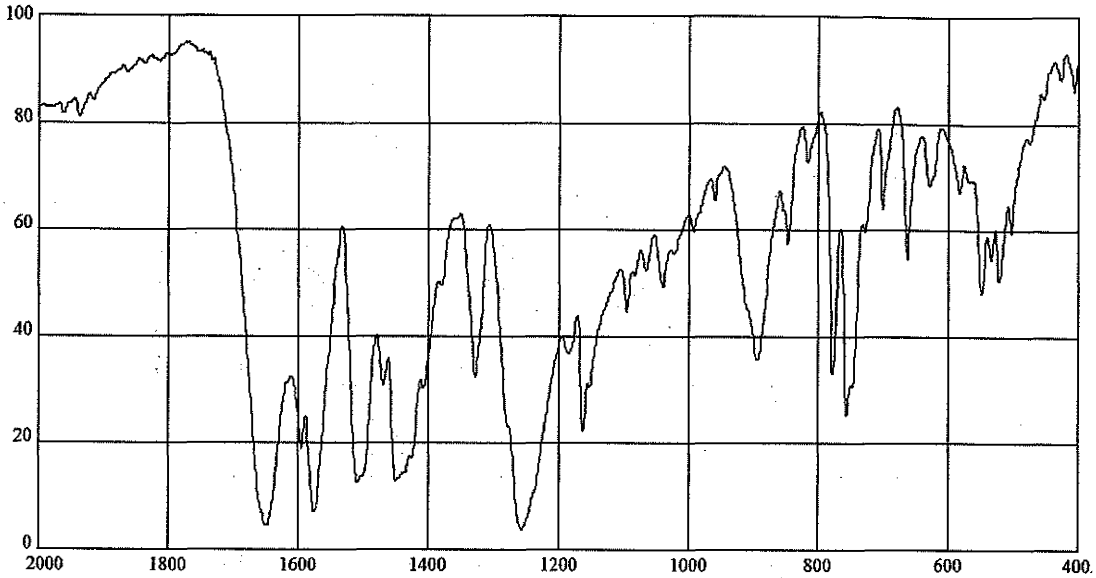
Phase: KBr disc



Mefenamic Acid

FTIR

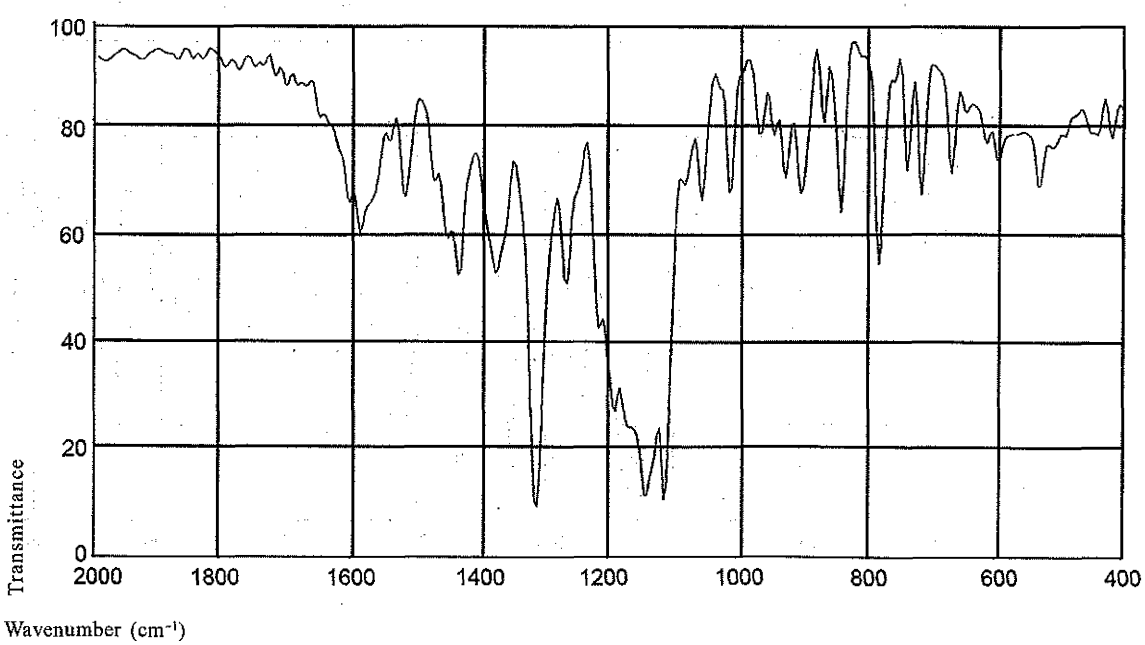
Phase: Liquid paraffin mull



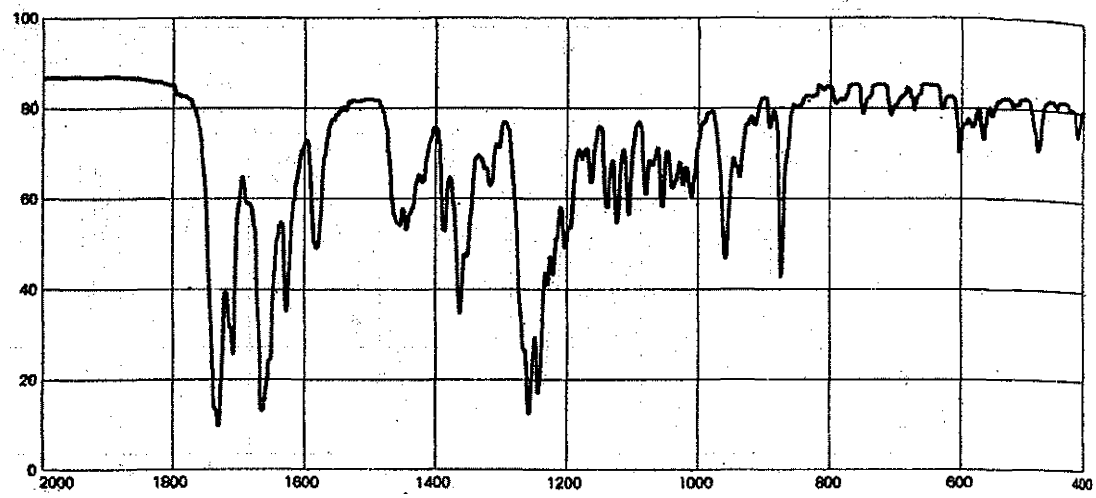
Mefloquine Hydrochloride

FTIR

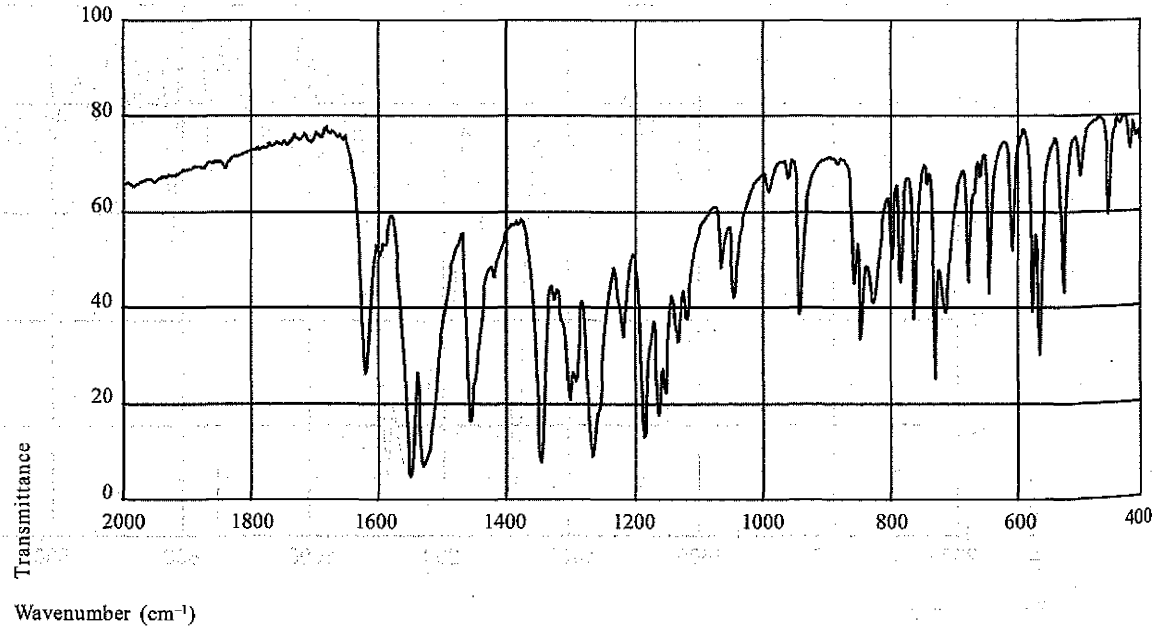
Phase: KBr disc



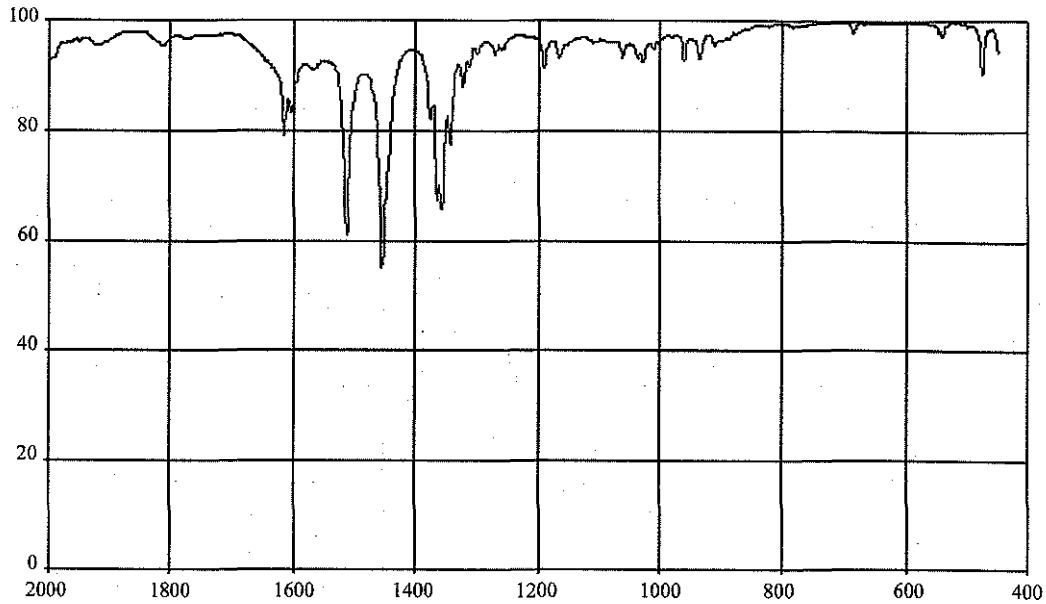
Megestrol Acetate *Dispersive IR* Phase: KBr disc



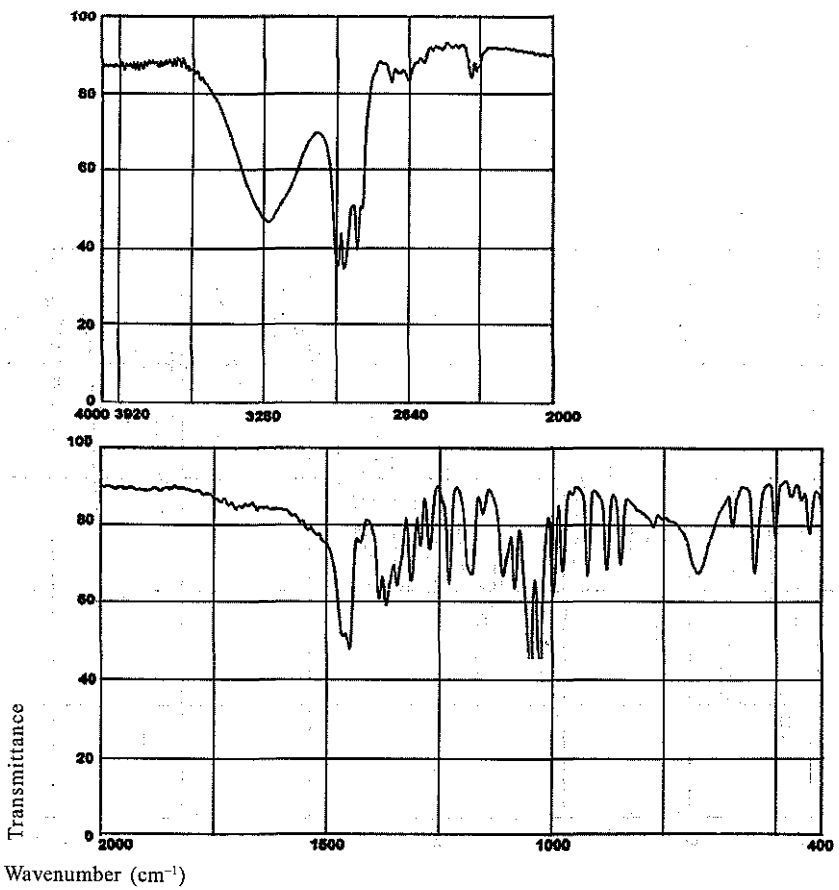
Meloxicam *FTIR* Phase: KBr disc



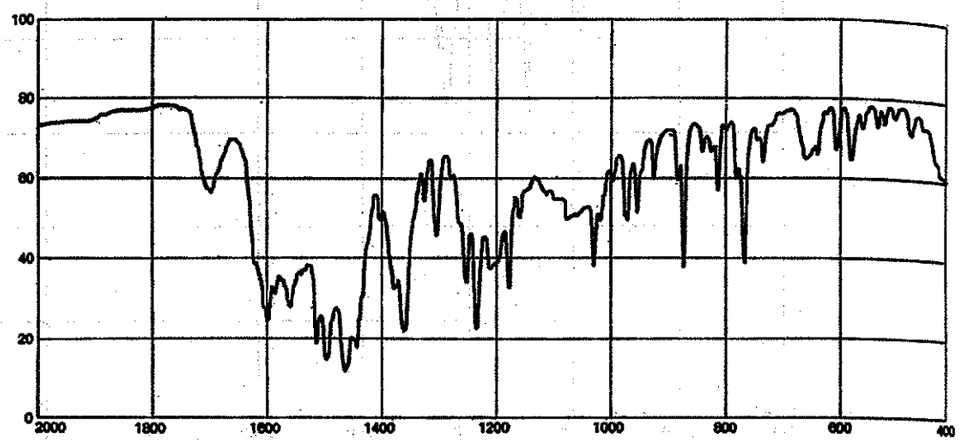
Memantine Hydrochloride *FTIR* Phase: KBr disc



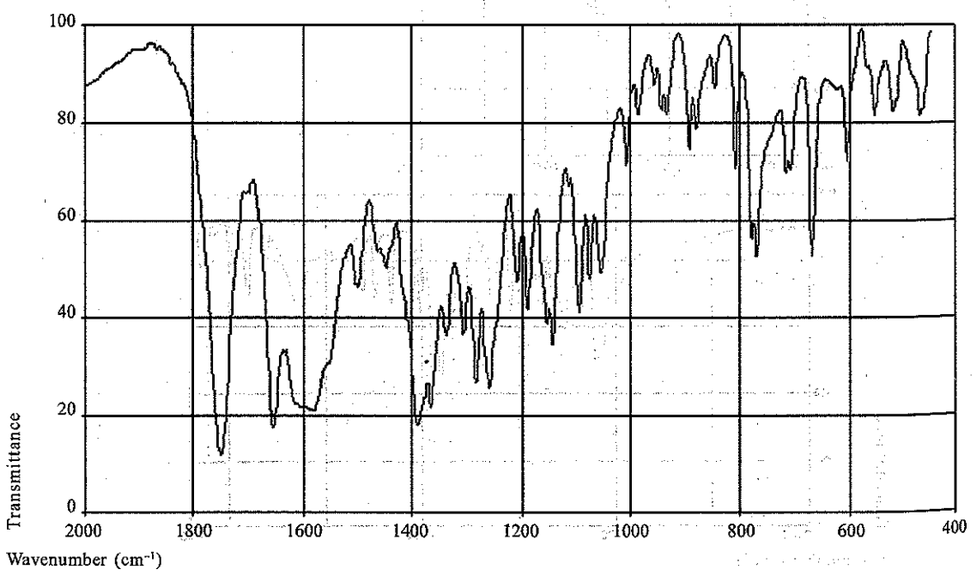
Menthol *FTIR* Phase: KBr disc



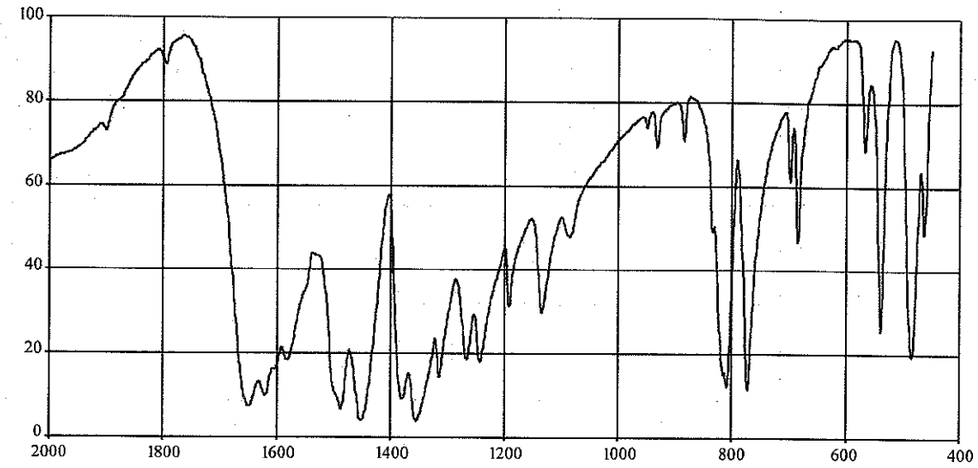
Mepyramine Maleate *Dispersive IR* Phase: KBr disc



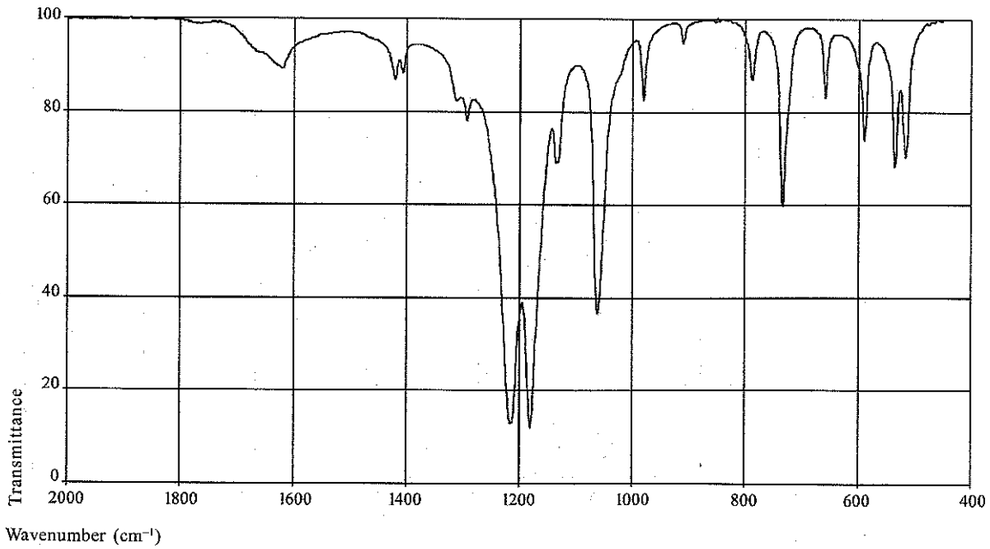
Meropenem *FTIR* Phase: KBr disc



Mesalazine *FTIR* Phase: KBr disc



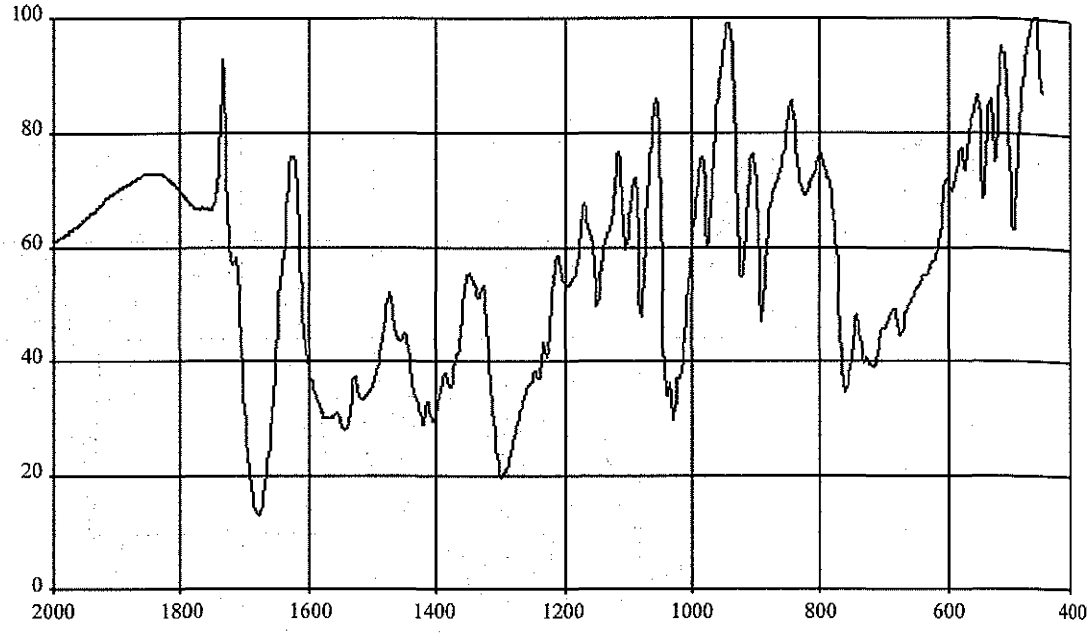
Mesna *FTIR* Phase: KCl disc



Metadoxine

FTIR

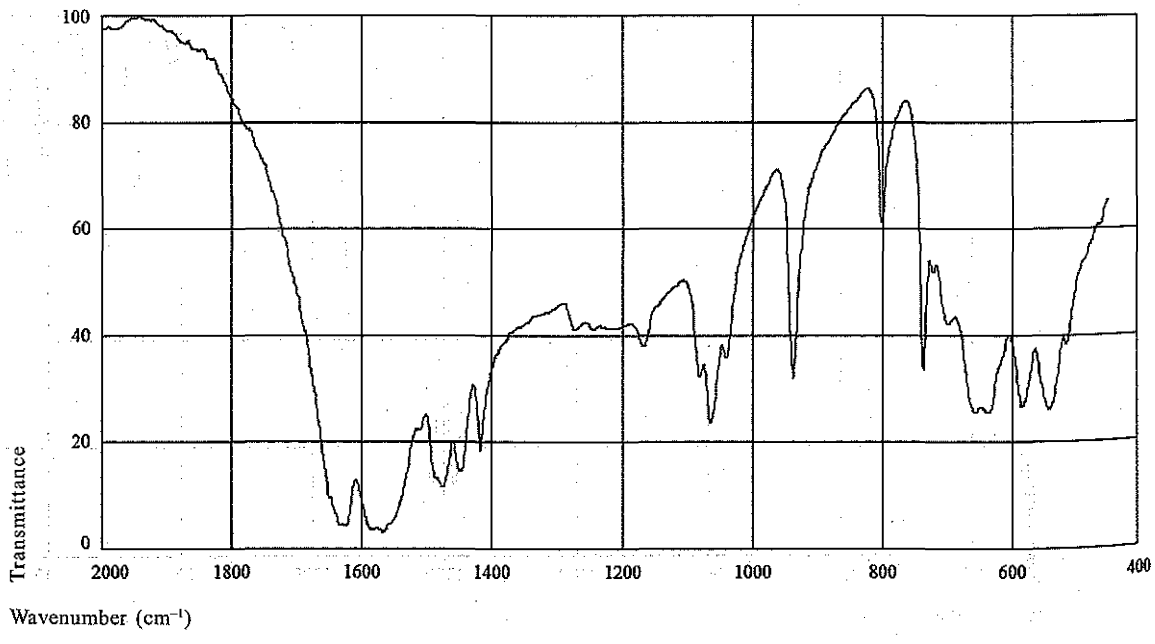
Phase: KCl disc



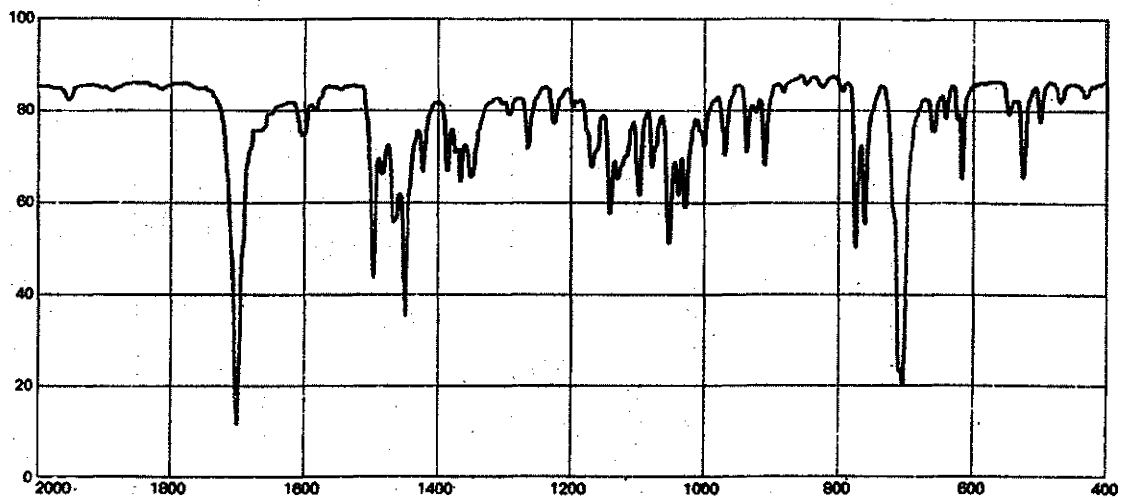
Metformin Hydrochloride

FTIR

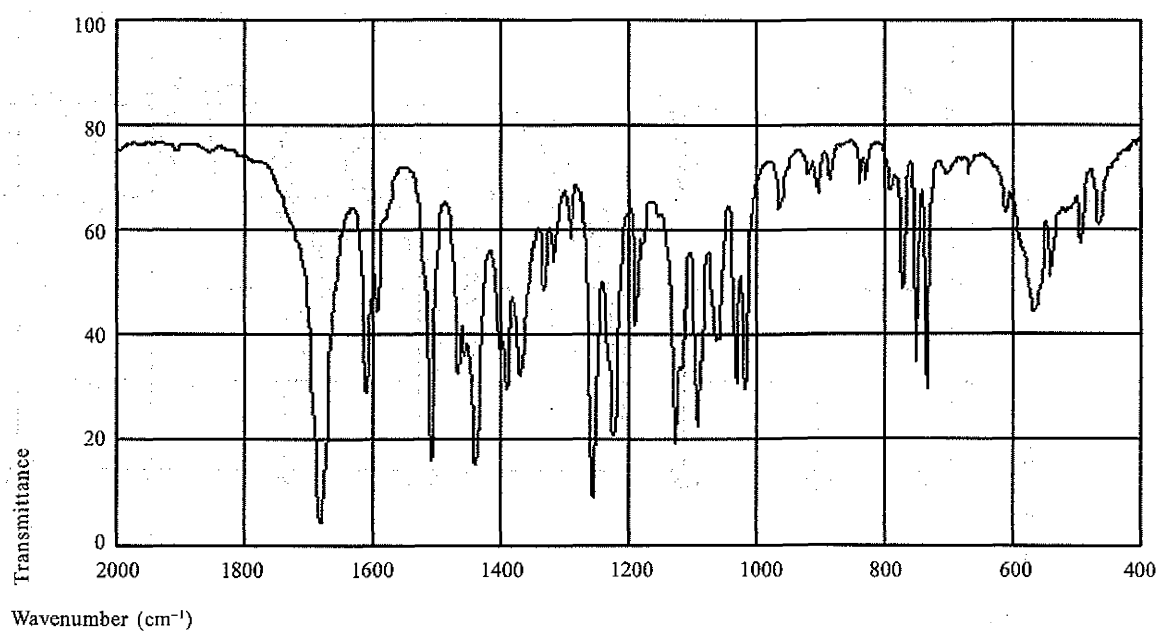
Phase: KCl disc



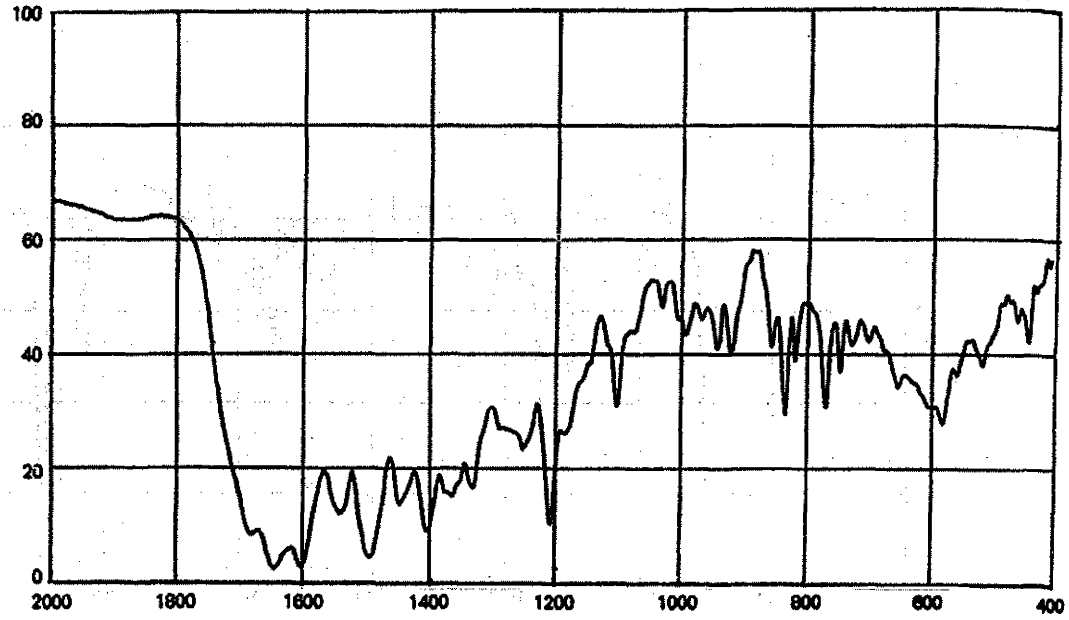
Methadone *Dispersive IR* Phase: KBr disc



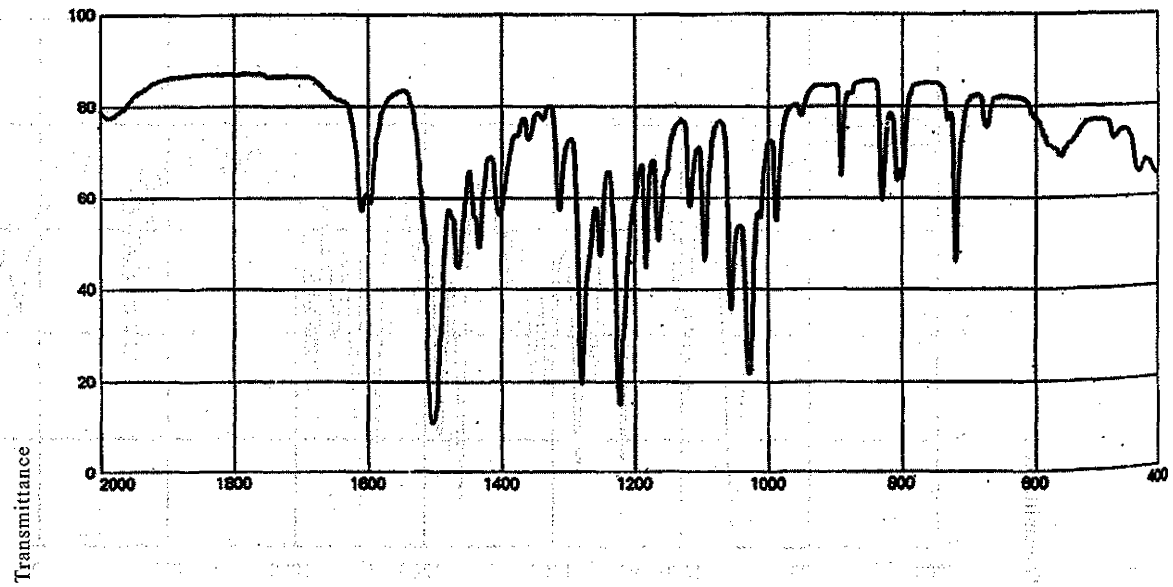
Methocarbamol *FTIR* Phase: KBr disc



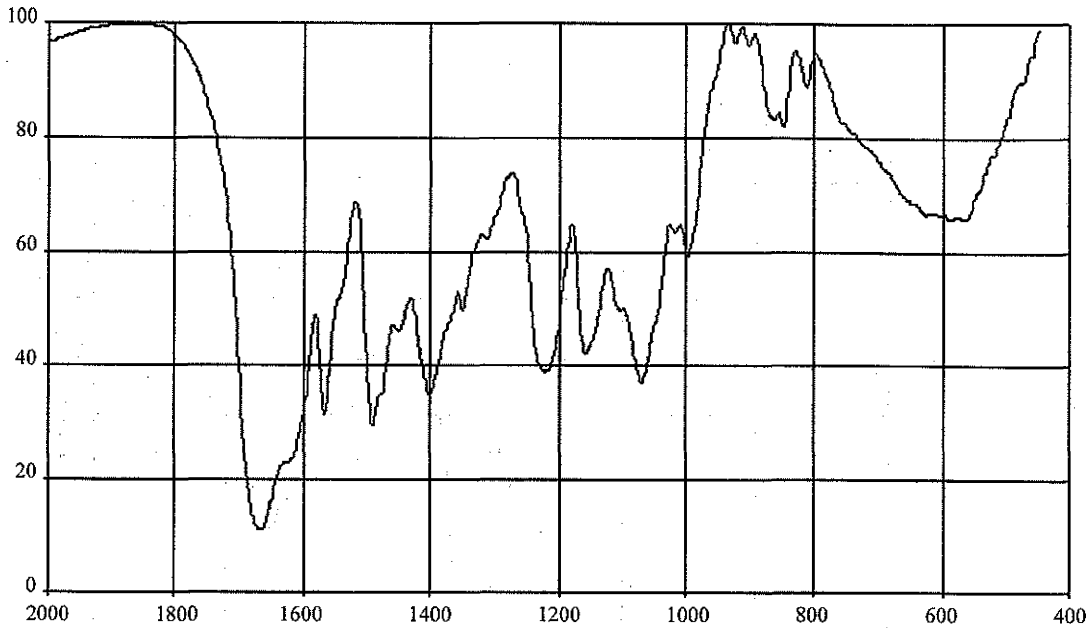
Methotrexate *FTIR* Phase: KBr disc



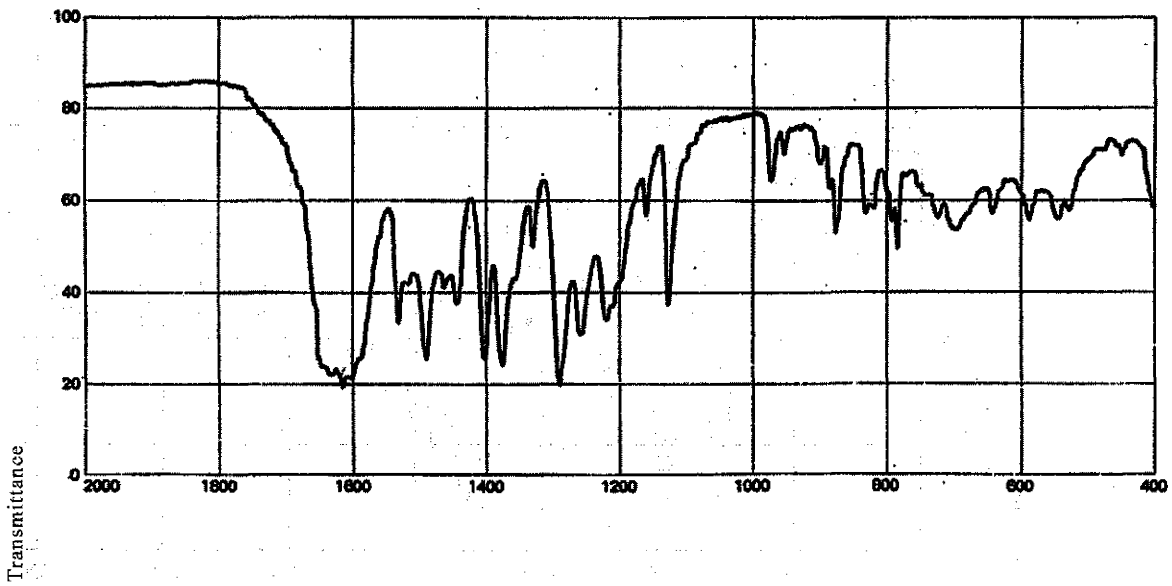
Methoxamine Hydrochloride *Dispersive IR* Phase: KCl disc



Methylcobalamin *FTIR* Phase: KBr disc



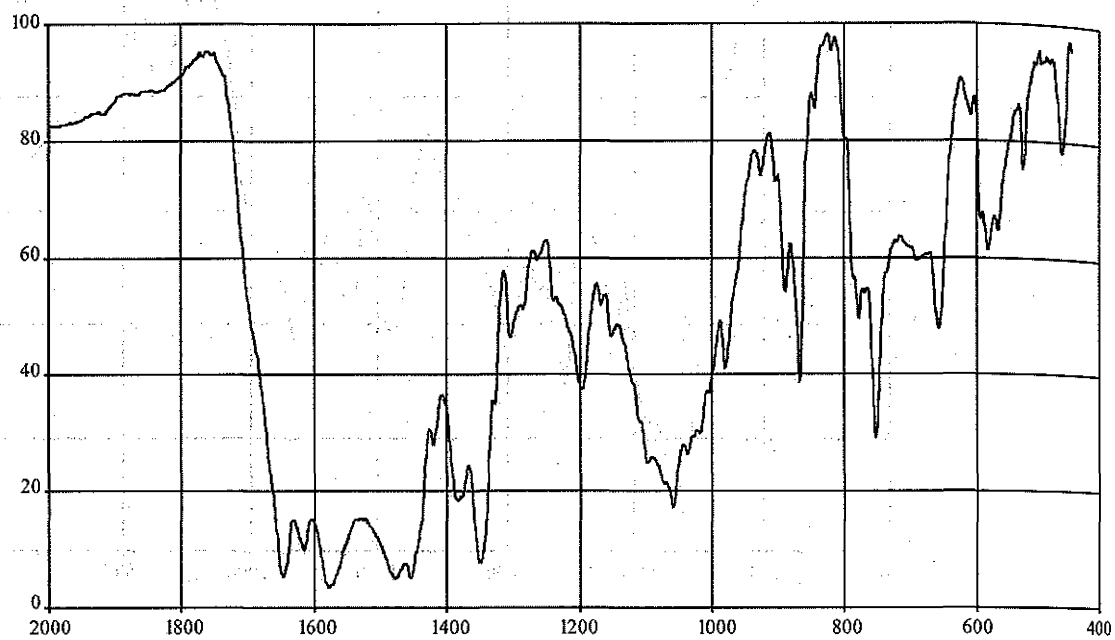
Methyldopa *Dispersive IR* Phase: KBr disc



Methylergometrine Maleate

FTIR

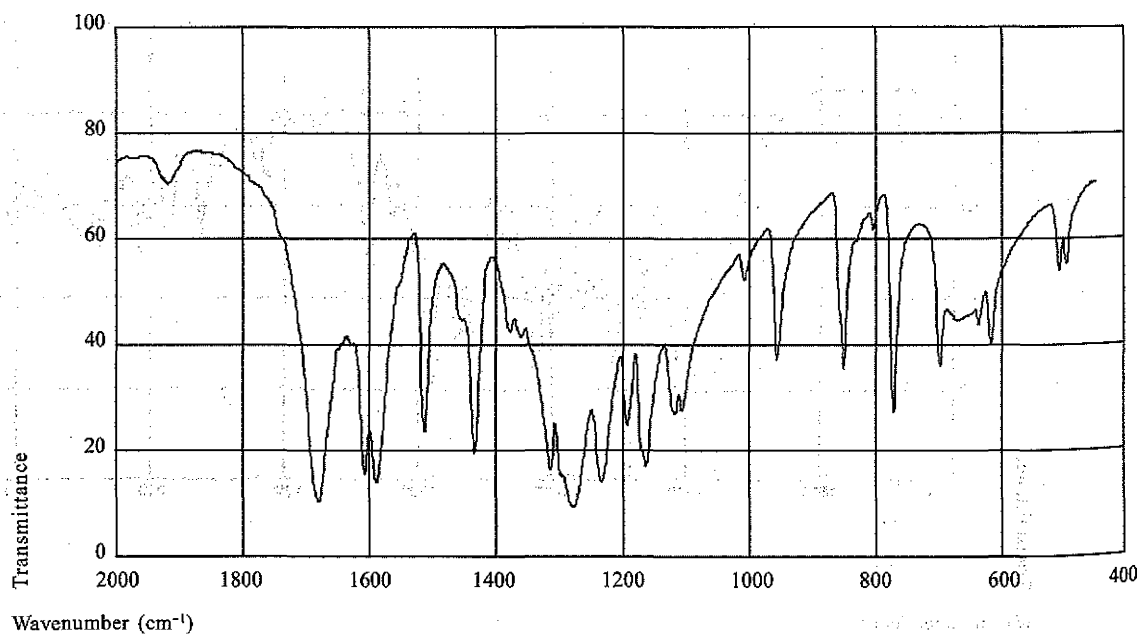
Phase: KBr disc



Methylparaben

FTIR

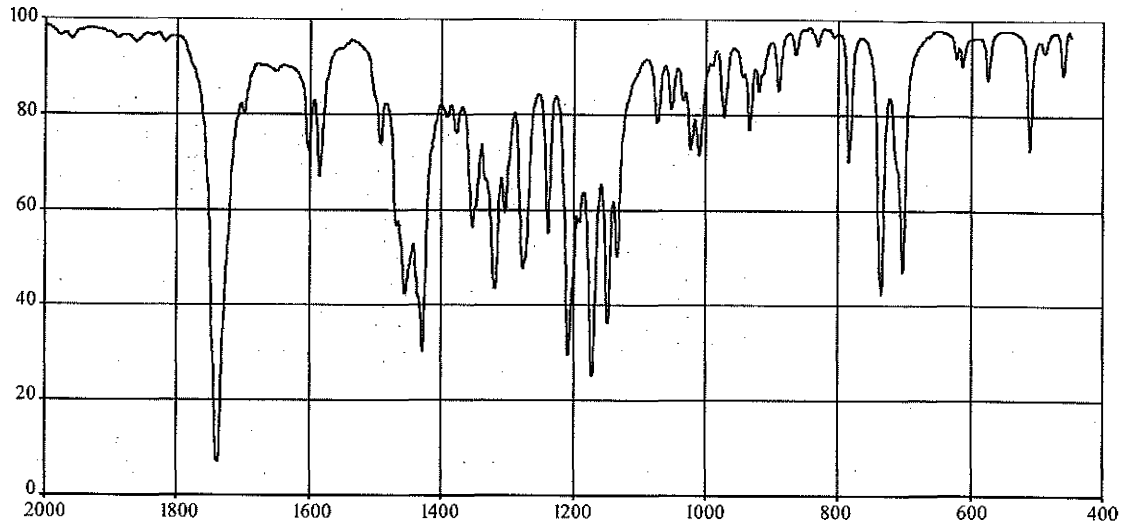
Phase: KBr disc



Methylphenidate Hydrochloride

FTIR

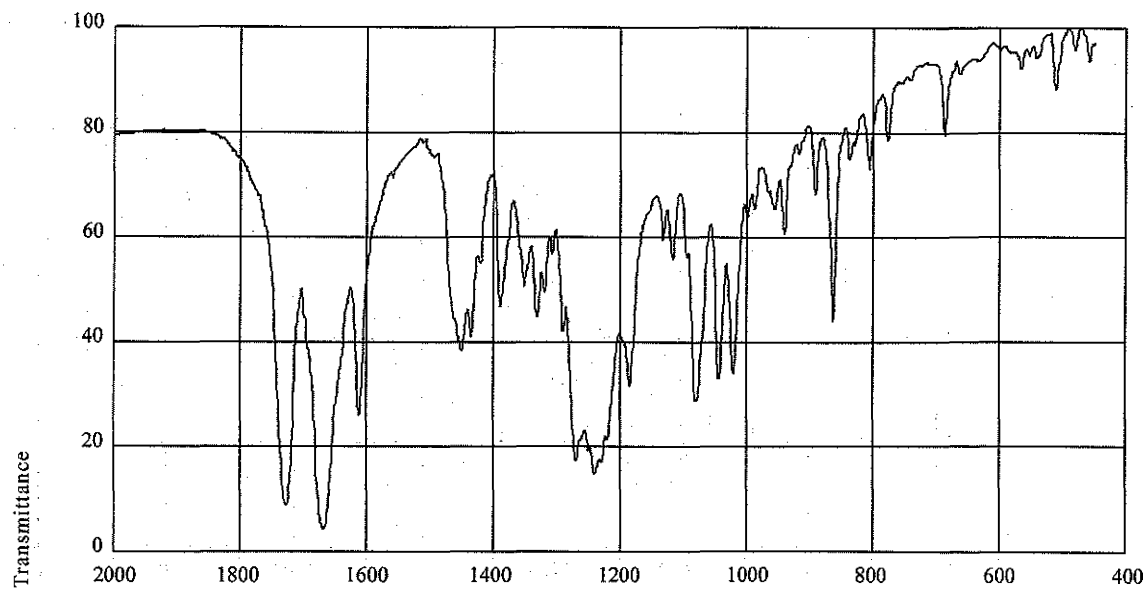
Phase: KBr disc



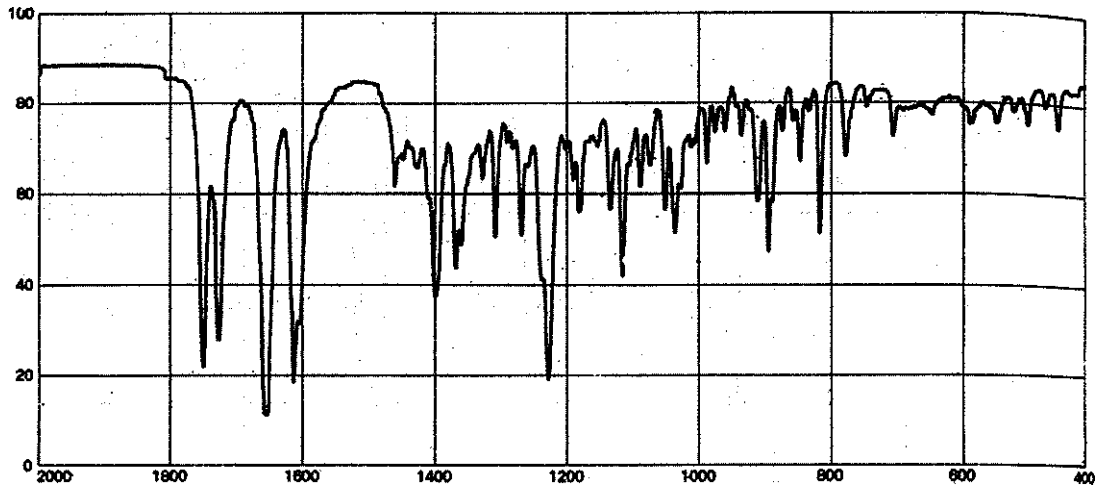
Methylprednisolone

FTIR

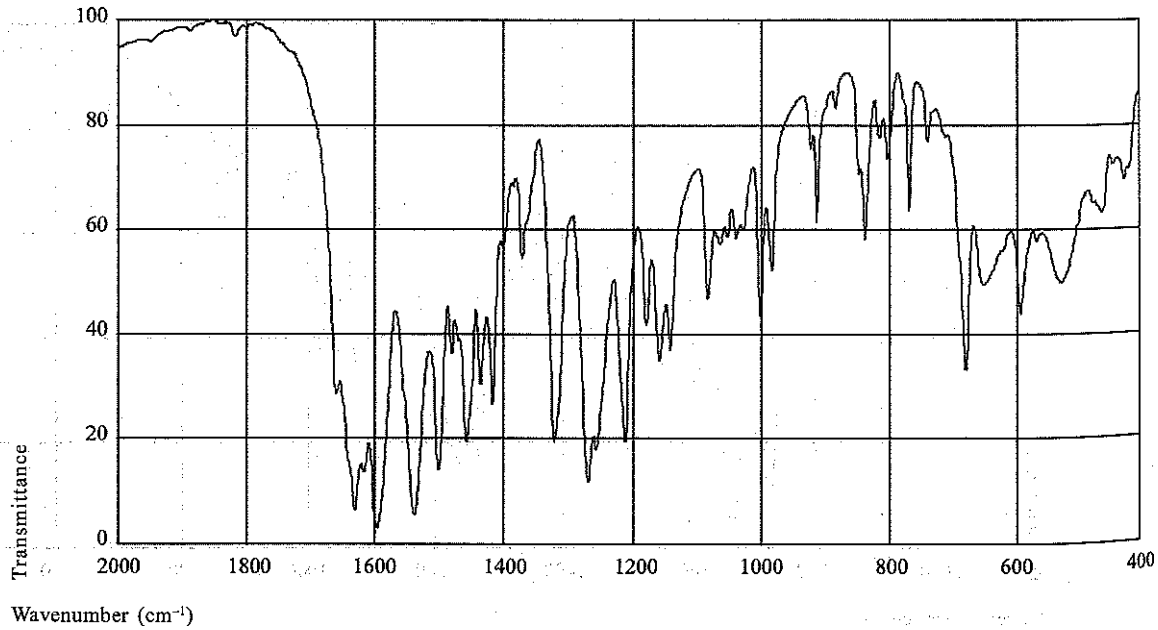
Phase: KBr disc



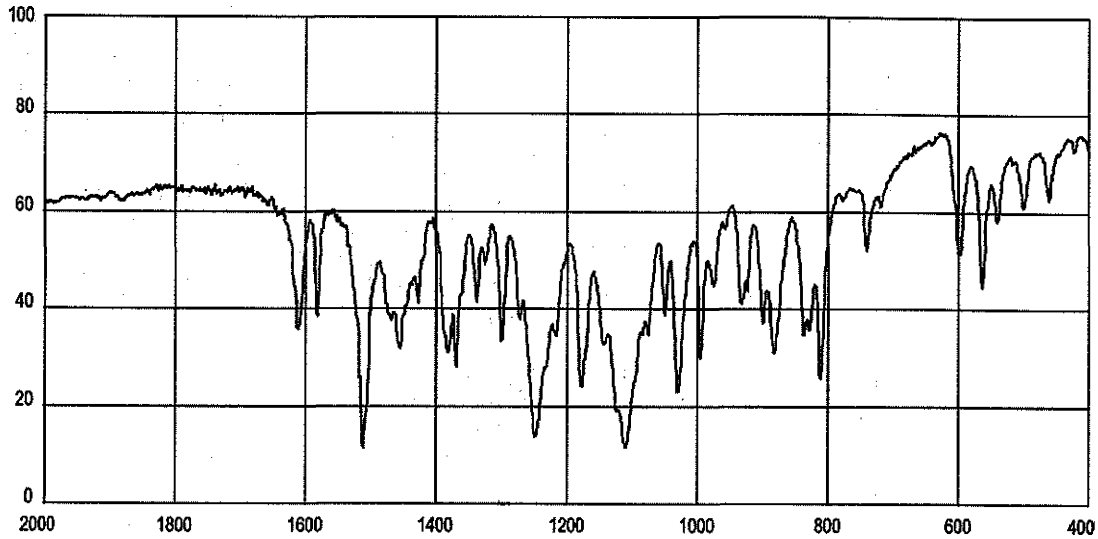
Methylprednisolone Acetate *Dispersive IR* Phase: KBr disc



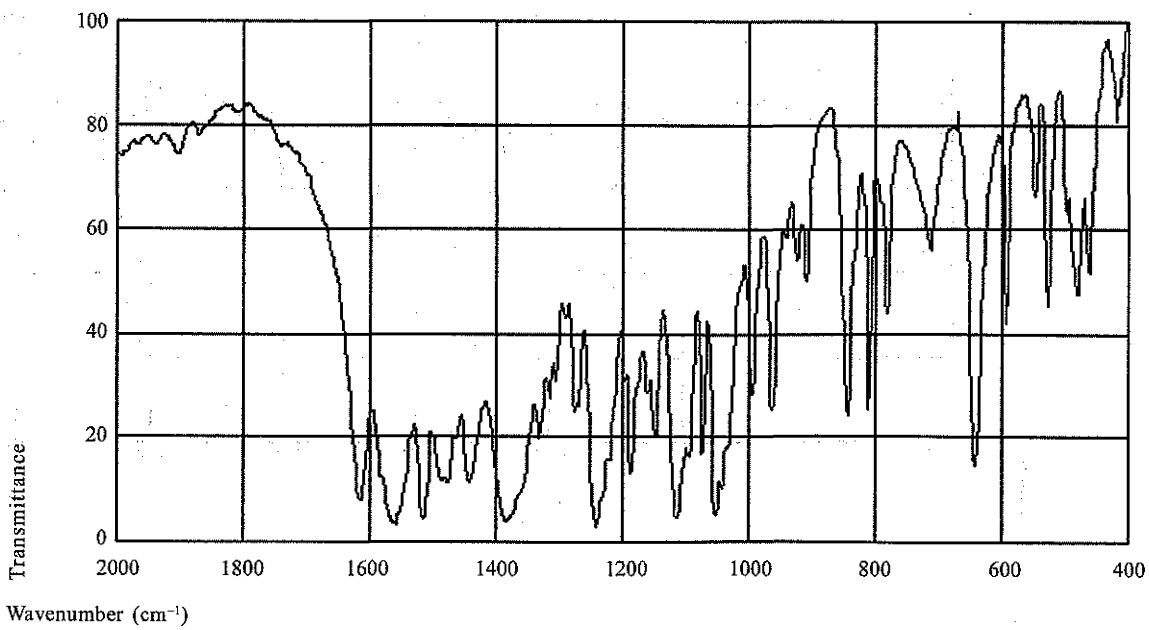
Metoclopramide Hydrochloride *FTIR* Phase: KCl disc



Metoprolol *FTIR* Phase: KCl disc



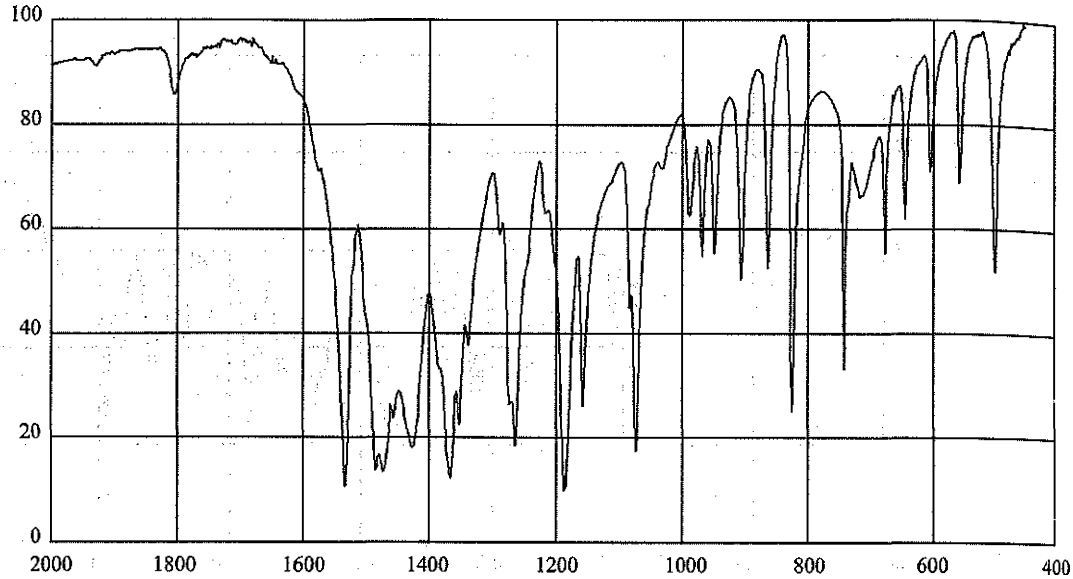
Metoprolol Succinate *FTIR* Phase: KBr disc



Metronidazole

FTIR

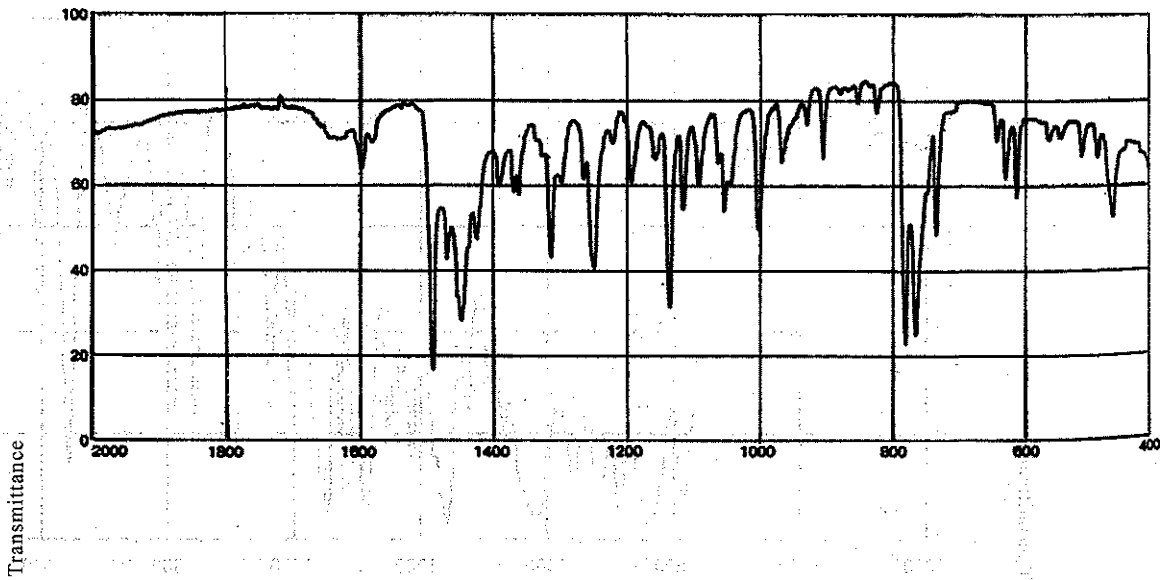
Phase: KBr disc



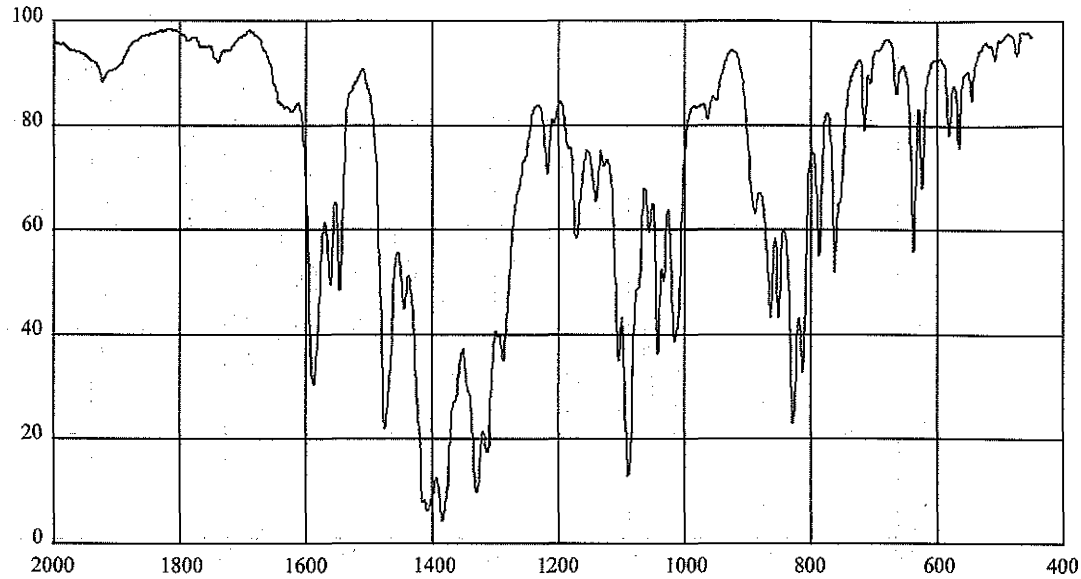
Mianserin Hydrochloride

Dispersive IR

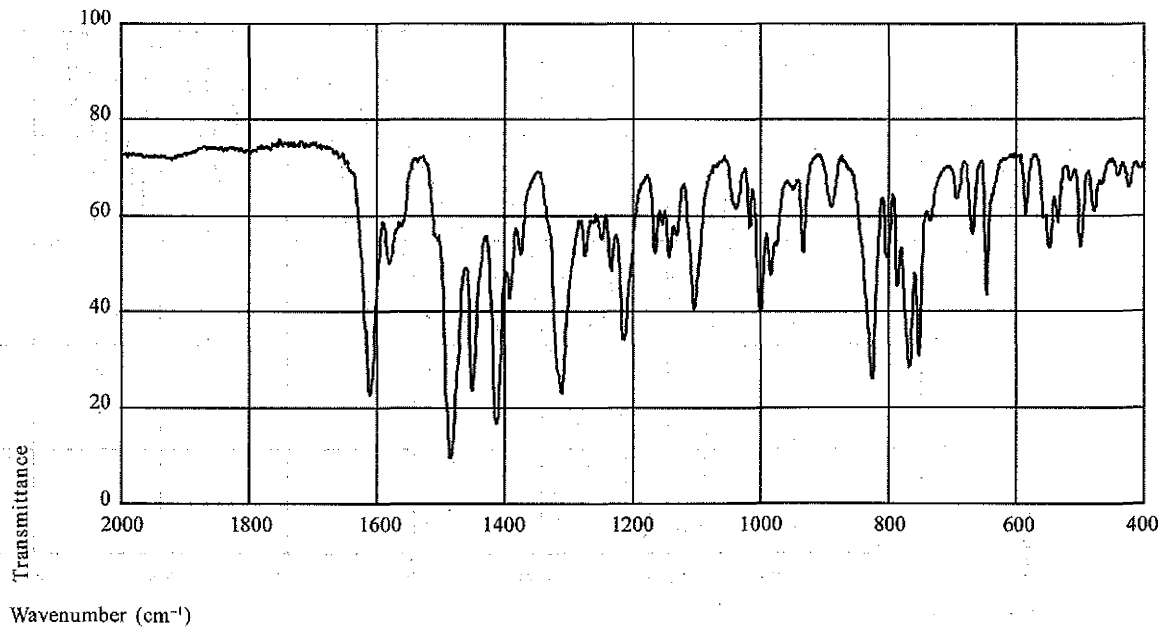
Phase: KCl disc



Miconazole Nitrate *FTIR* Phase: KBr disc



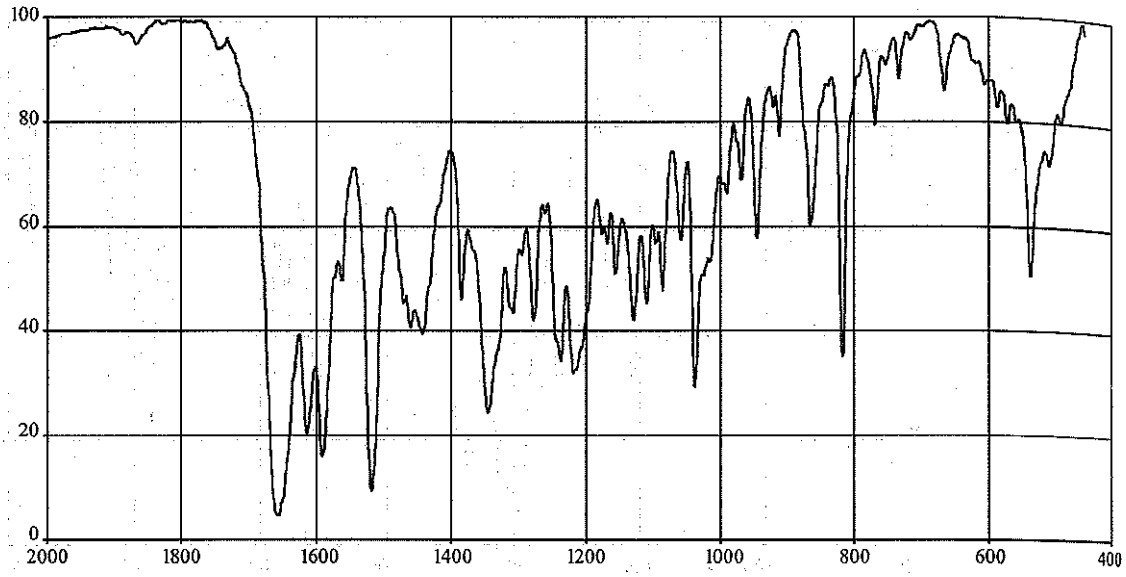
Midazolam *FTIR* Phase: KBr disc



Mifepristone

FTIR

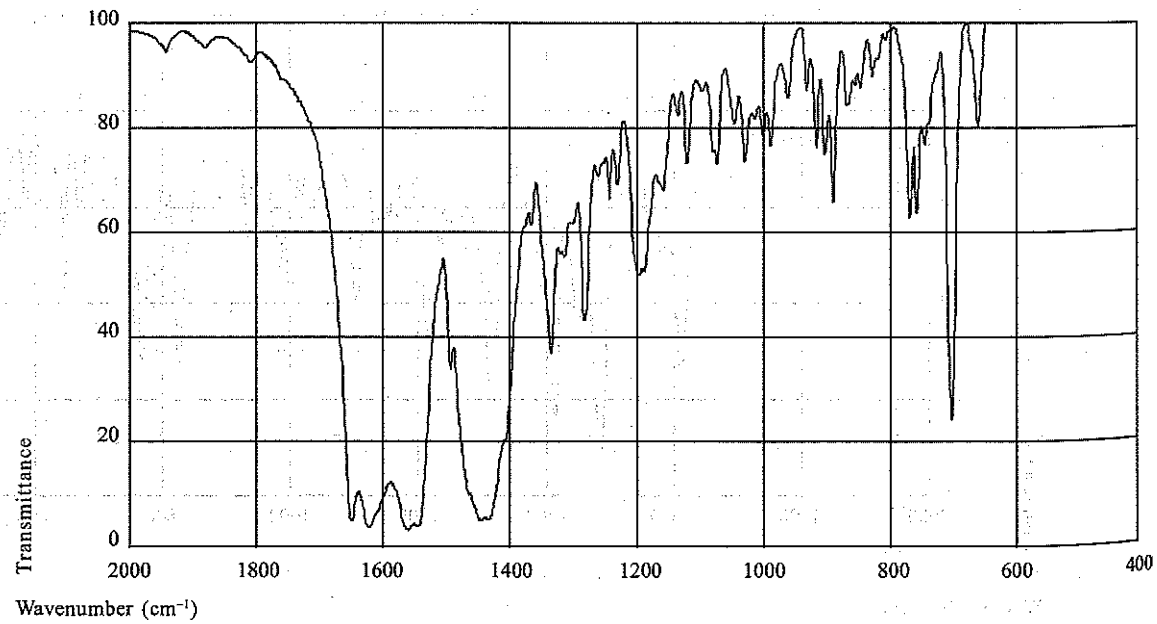
Phase: KBr disc



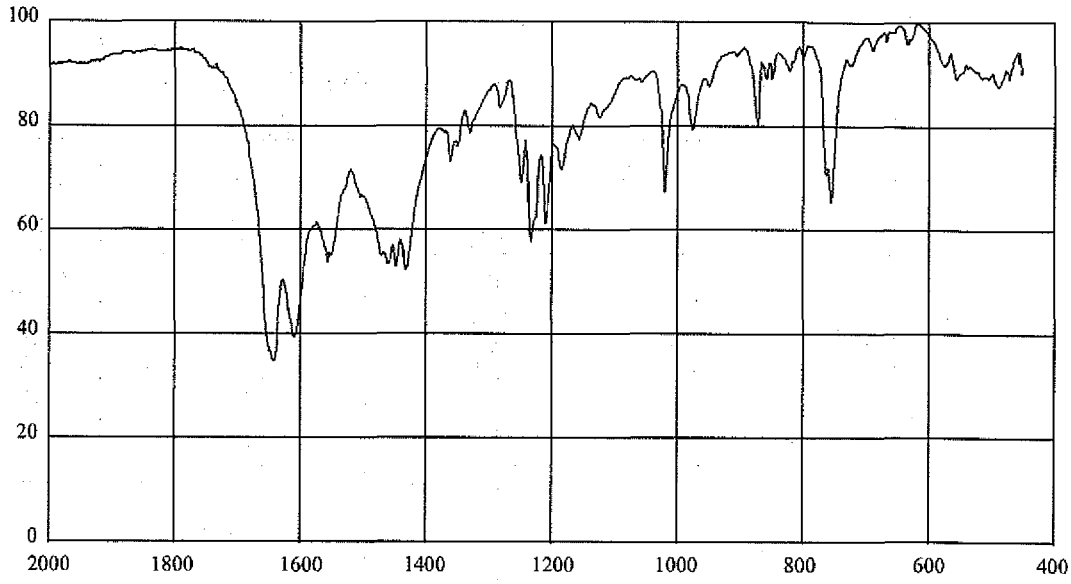
Mitiglinide Calcium Dihydrate

FTIR

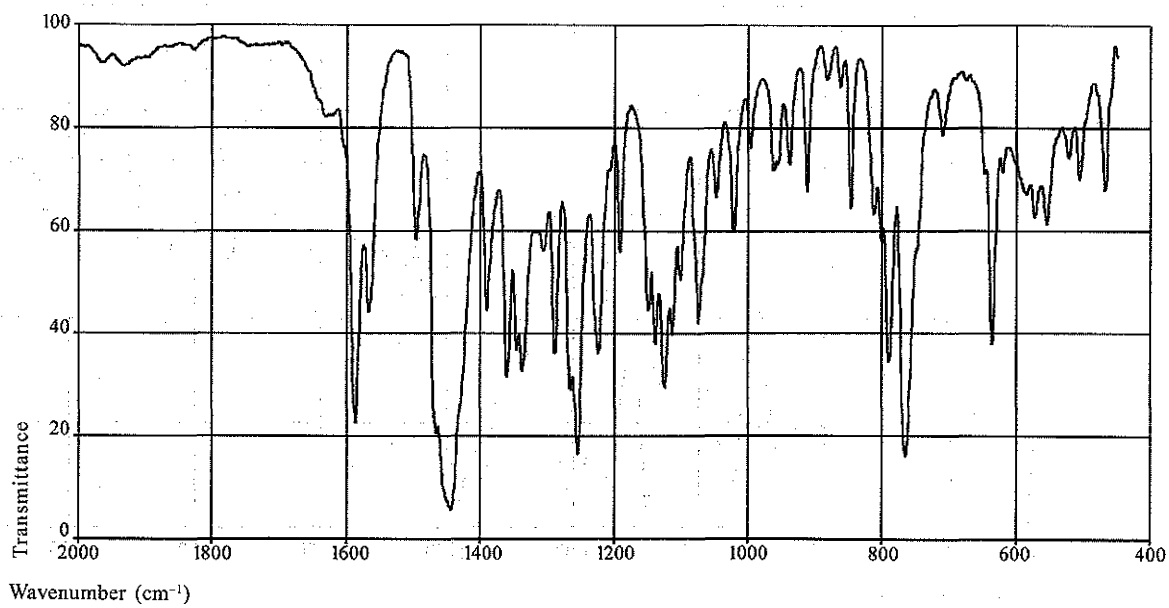
Phase: KBr disc



Minoxidil *FTIR* Phase: KBr disc



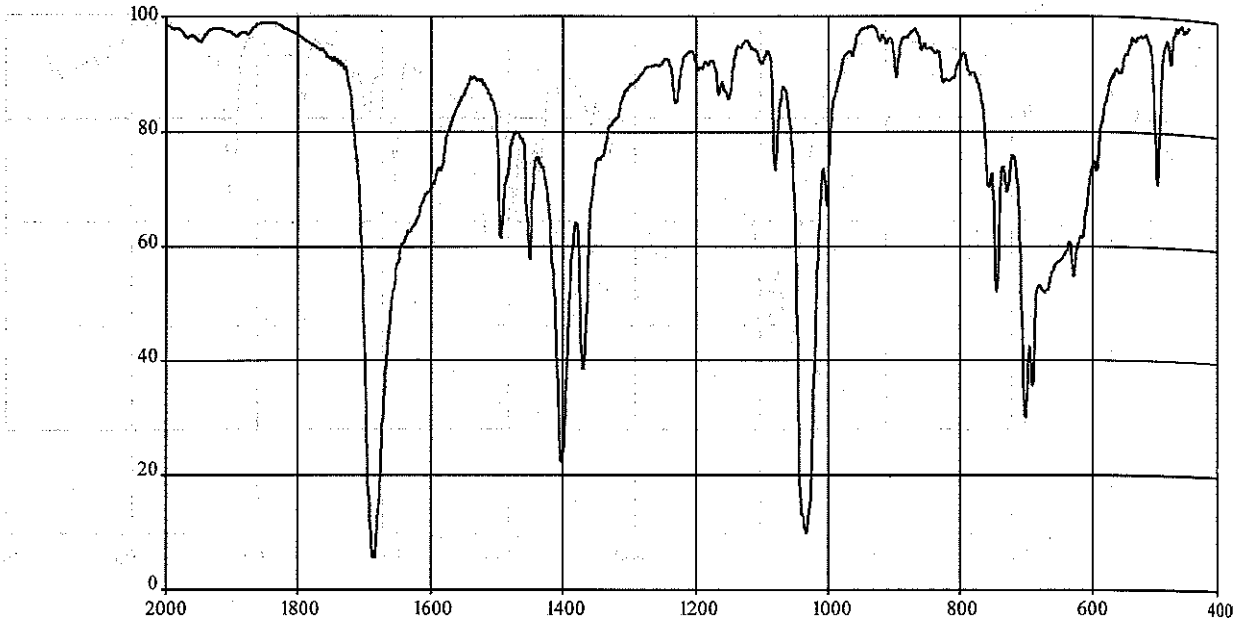
Mirtazapine *FTIR* Phase: KBr disc



Modafinil

FTIR

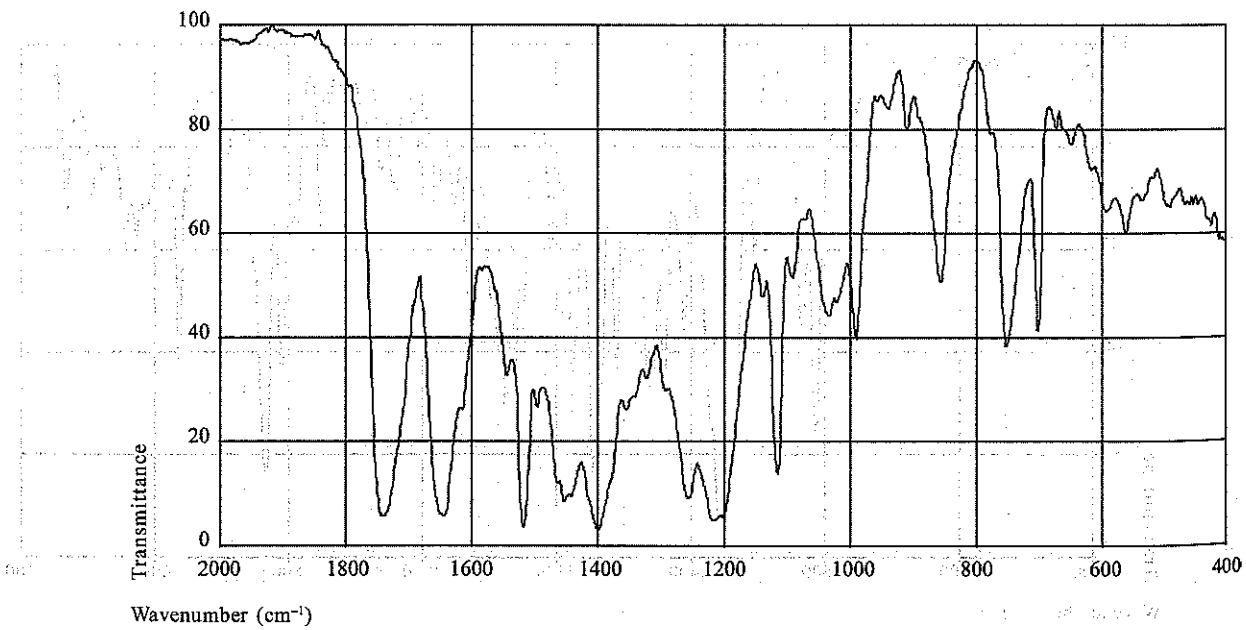
Phase: KBr disc



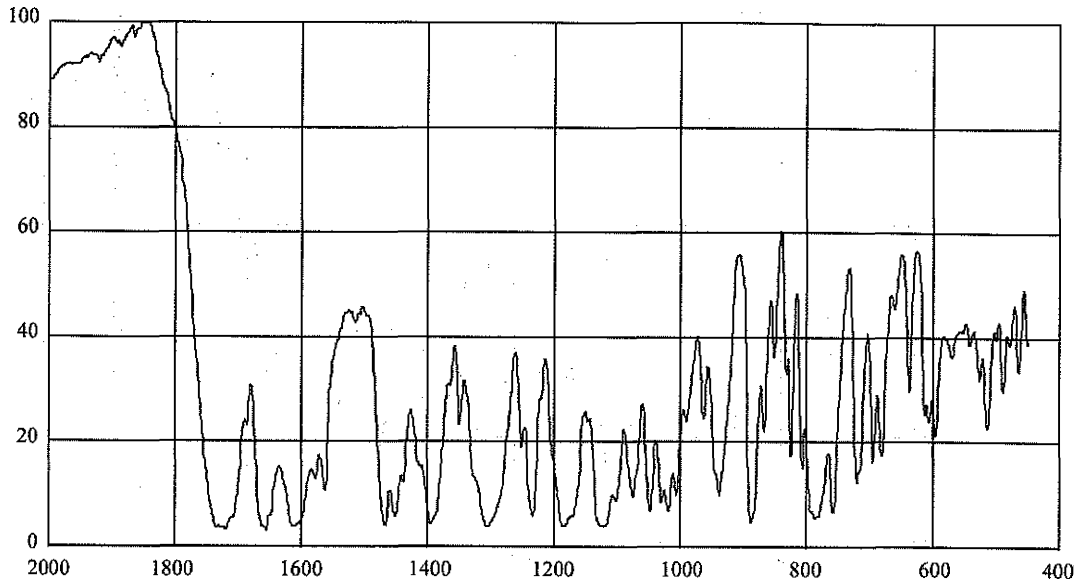
Moexipril Hydrochloride

FTIR

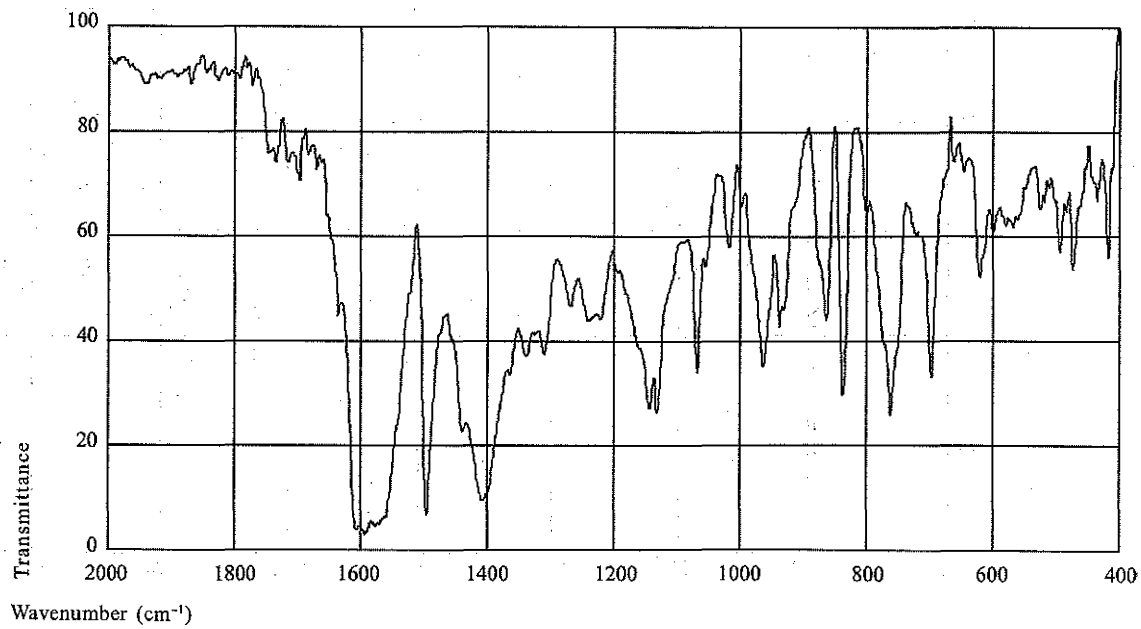
Phase: KBr disc



Mometasone Furoate *FTIR* Phase: KBr disc



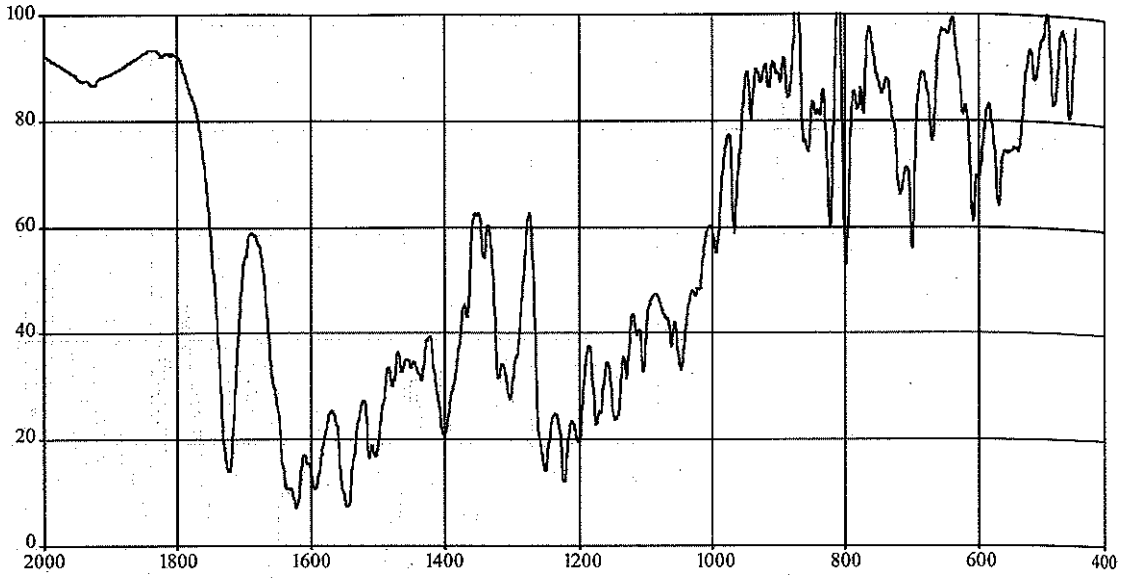
Montelukast Sodium *FTIR* Phase: KBr disc



Mosapride Citrate Dihydrate

FTIR

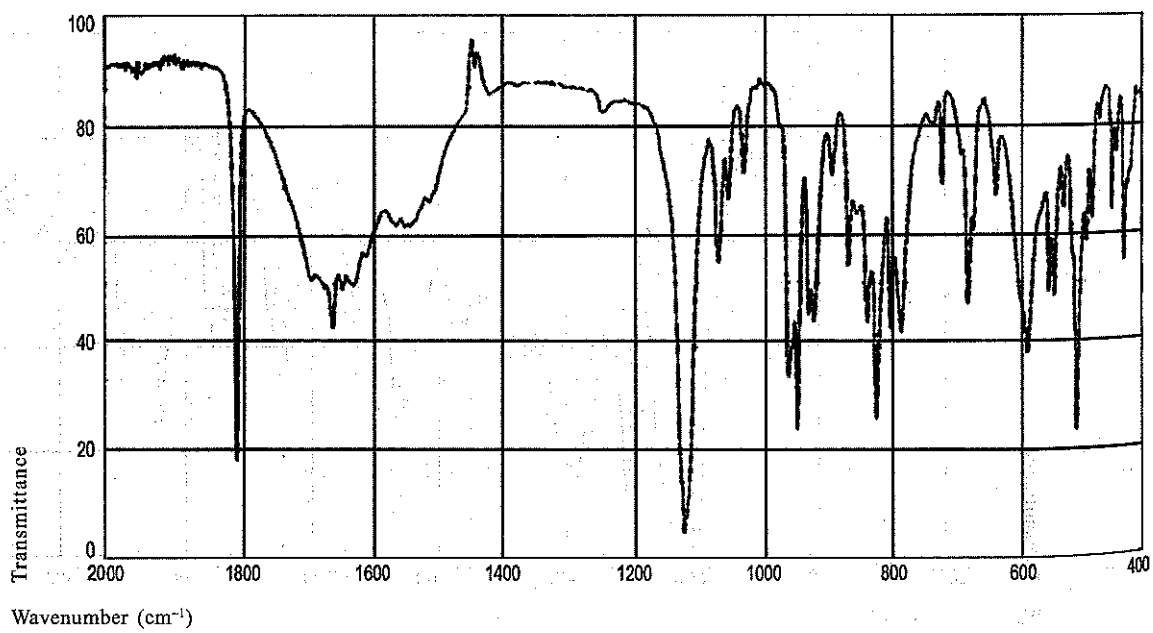
Phase: KBr disc



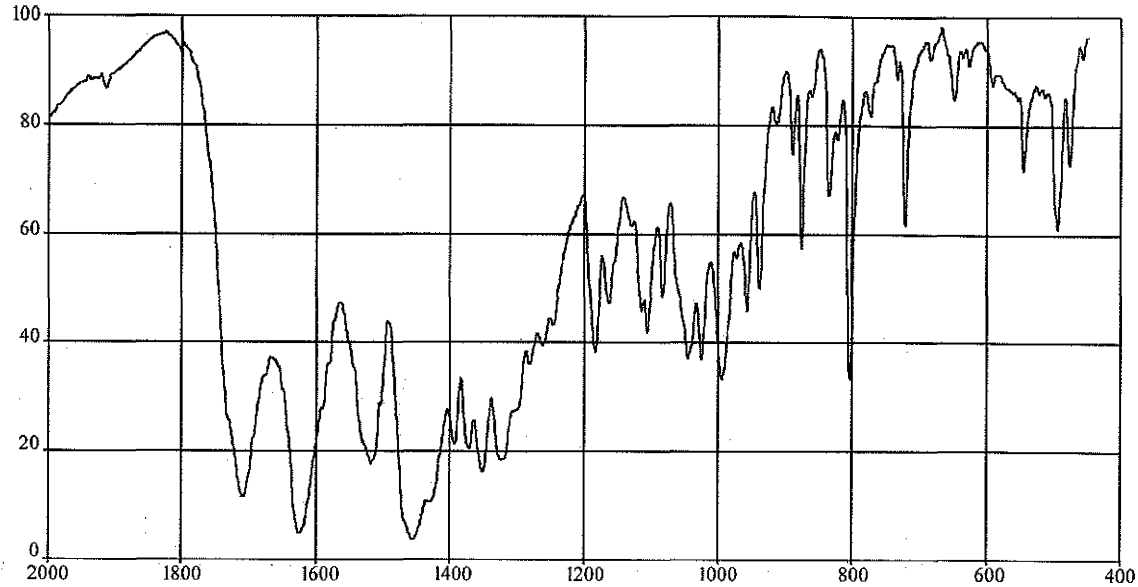
Moxidectin

FTIR

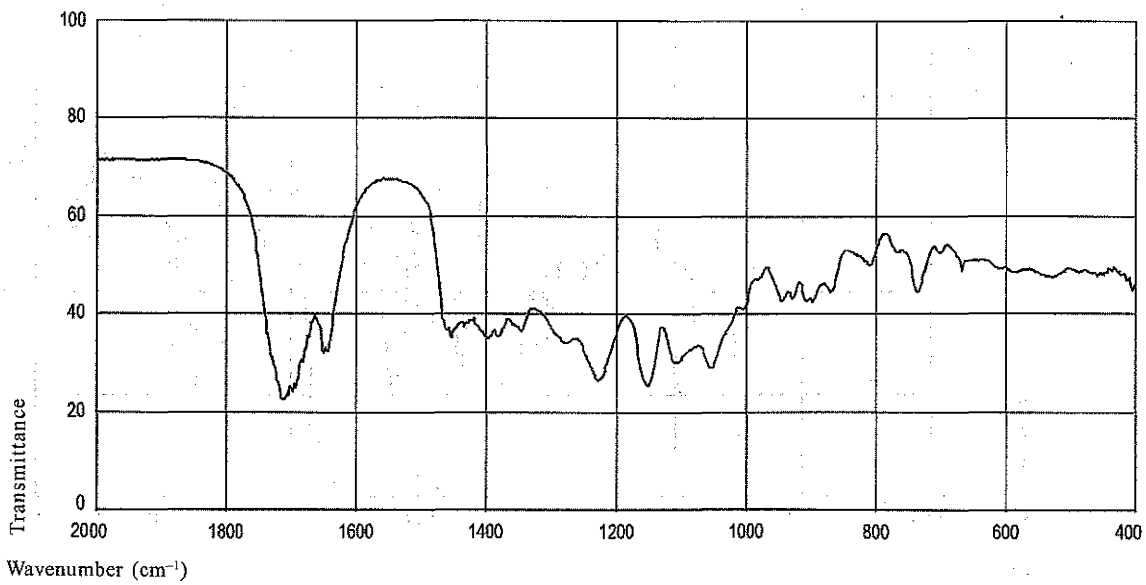
Phase: KBr disc



Moxifloxacin Hydrochloride *FTIR* Phase: KBr disc



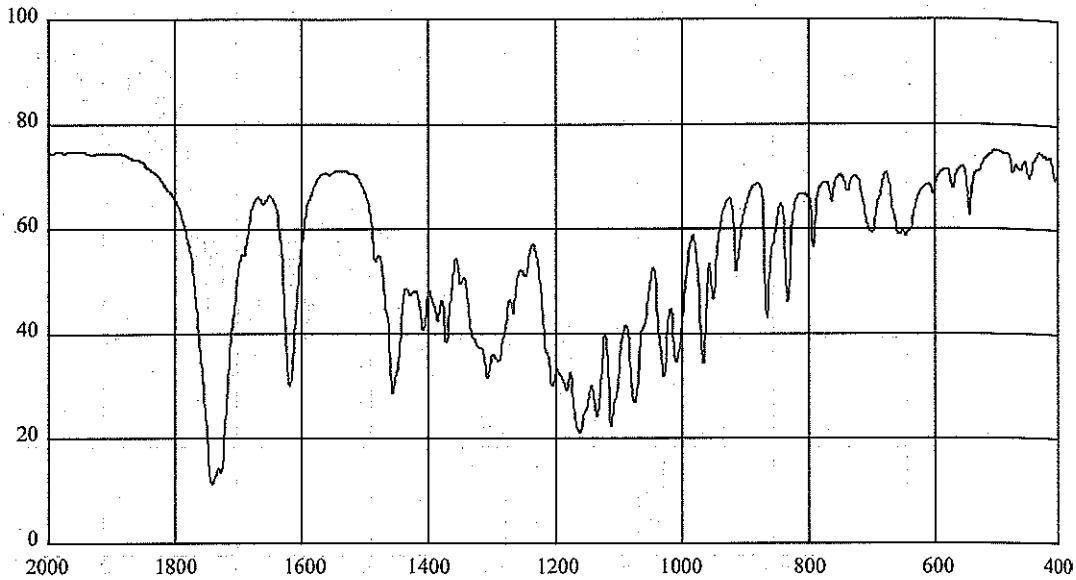
Mupirocin *FTIR* Phase: KBr disc



Mycophenolate Mofetil

FTIR

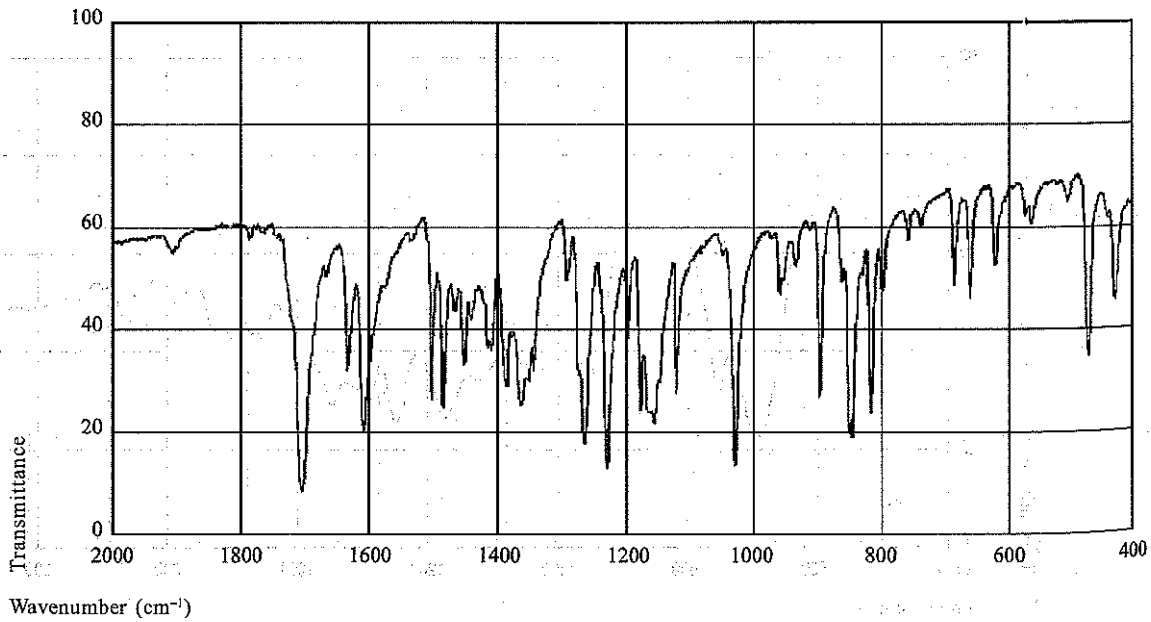
Phase: KBr disc



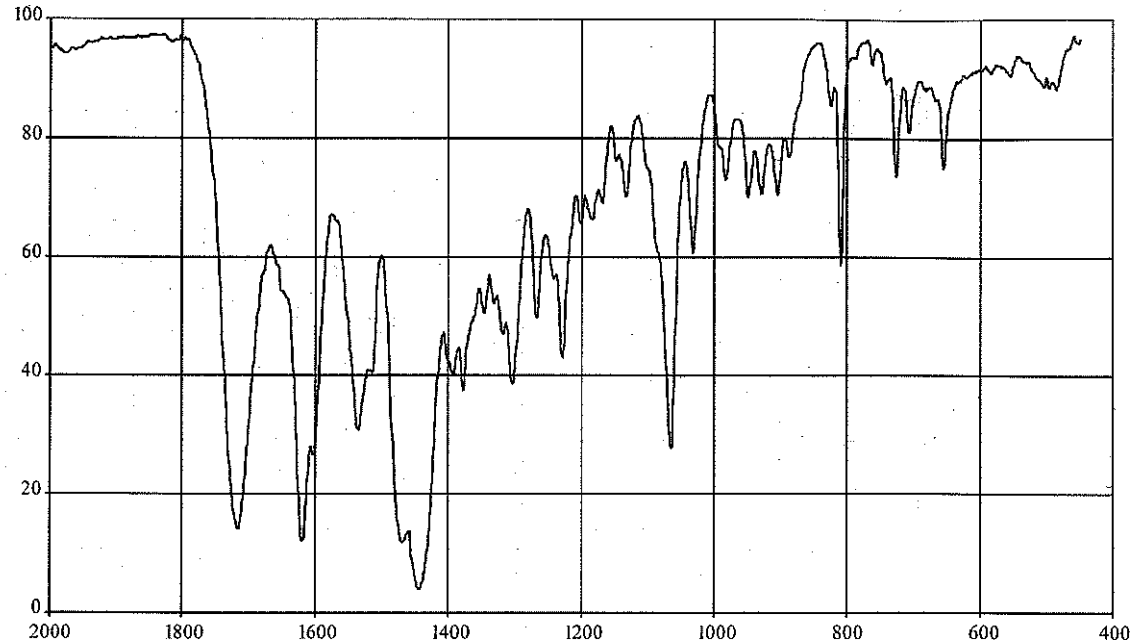
Nabumetone

FTIR

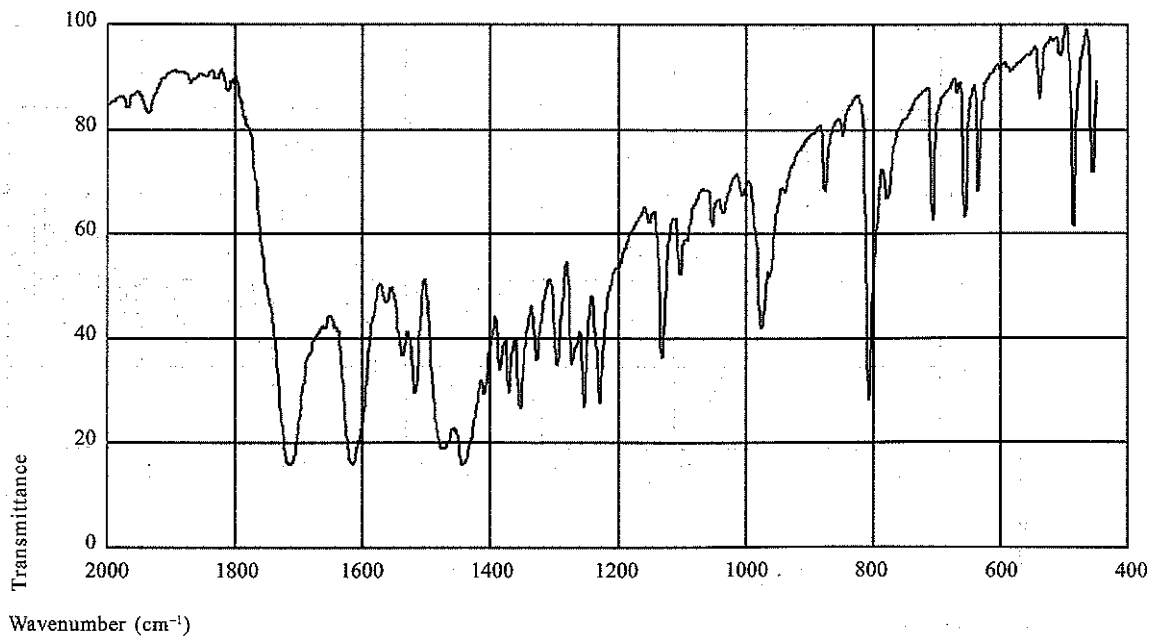
Phase: KBr disc



Nadifloxacin *FTIR* Phase: KBr disc



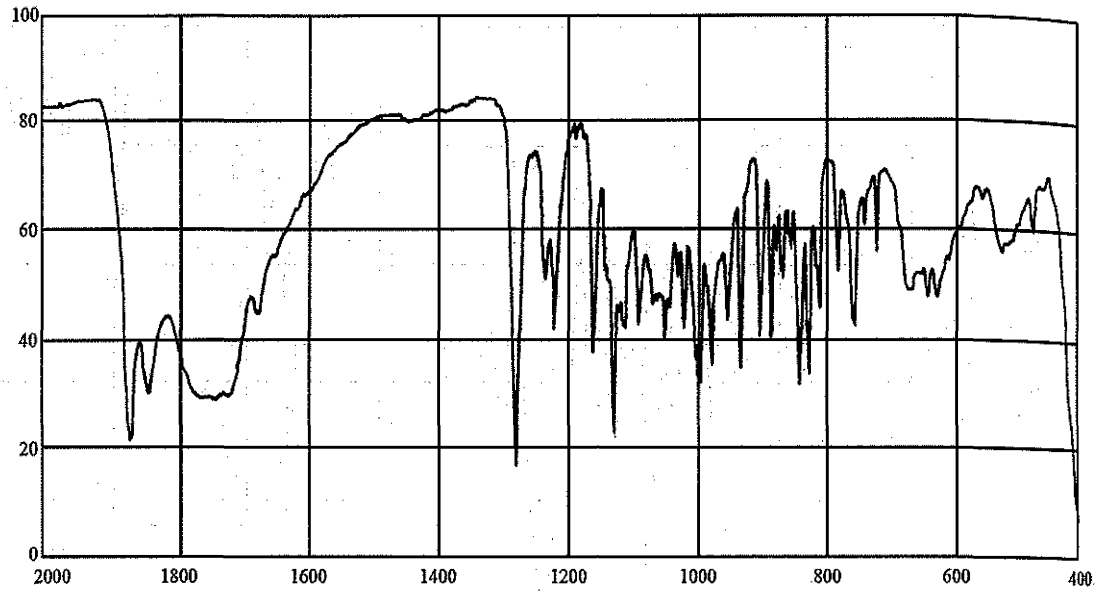
Nalidixic Acid *FTIR* Phase: KBr disc



Naloxone Hydrochloride

FTIR

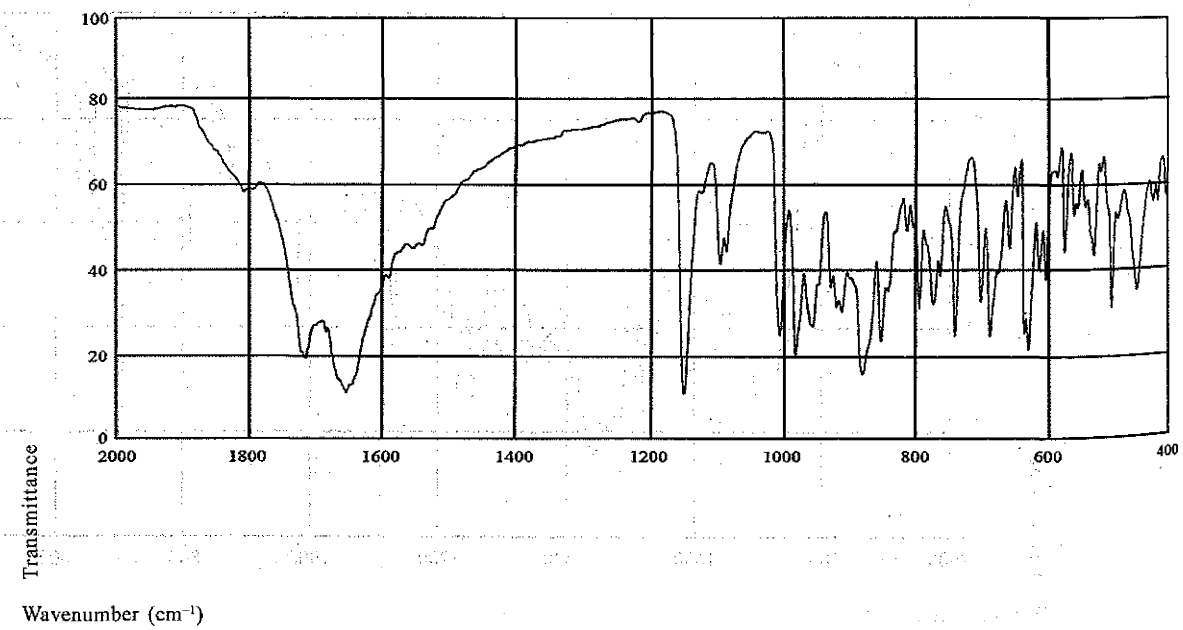
Phase: KBr disc



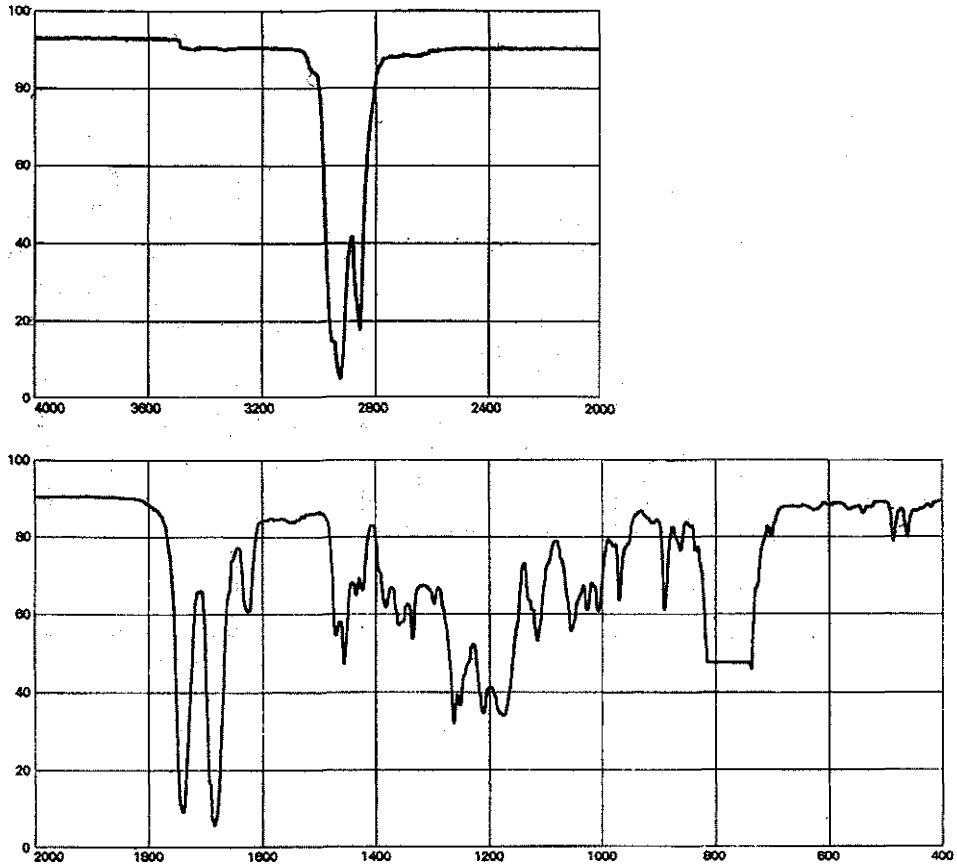
Naltrexone Hydrochloride

FTIR

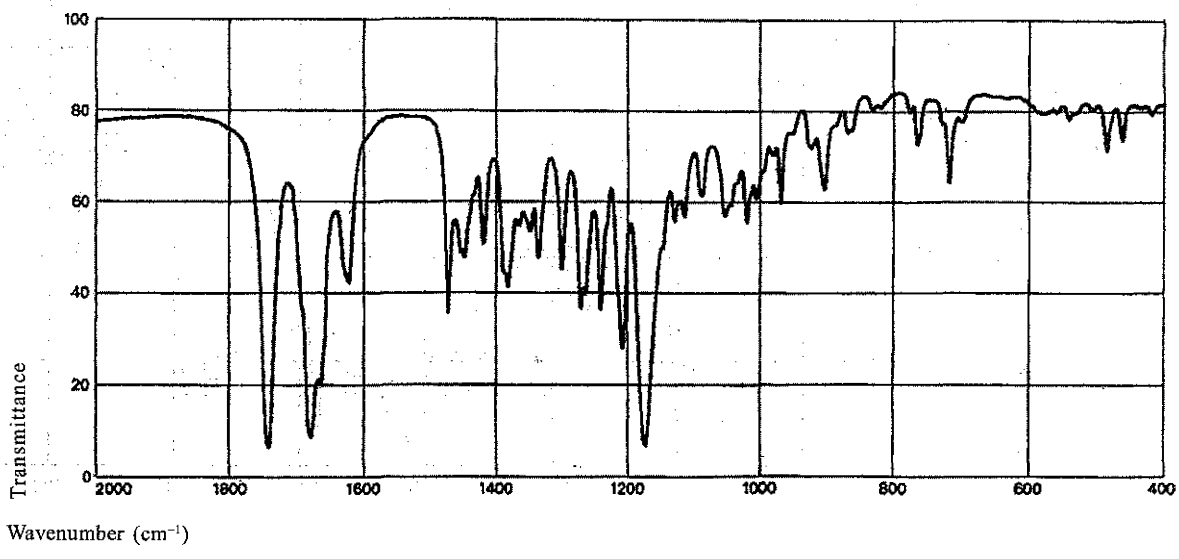
Phase: KBr disc



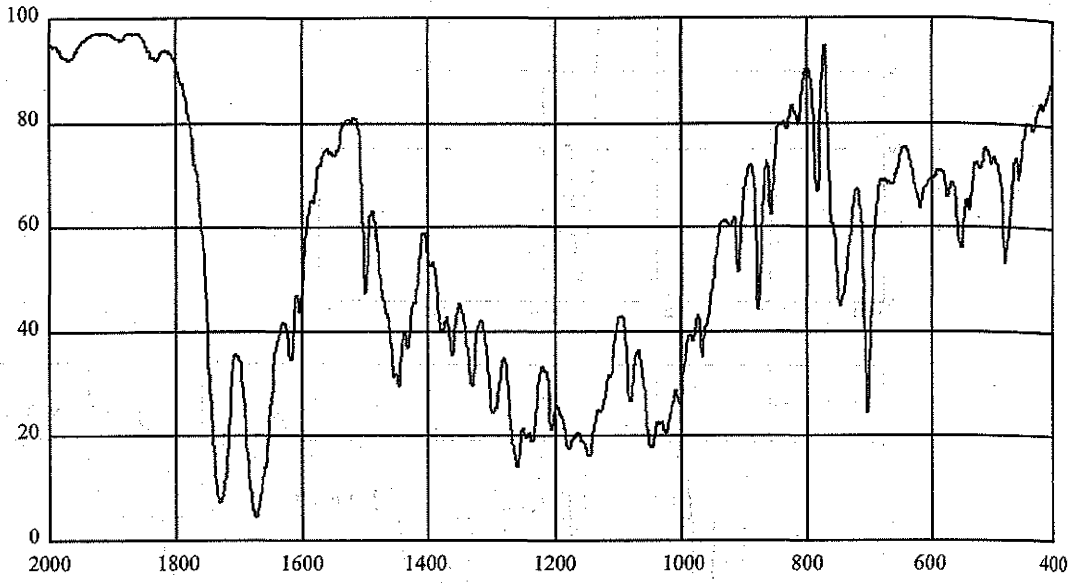
Nandrolone Decanoate *Dispersive IR* Phase: 10 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



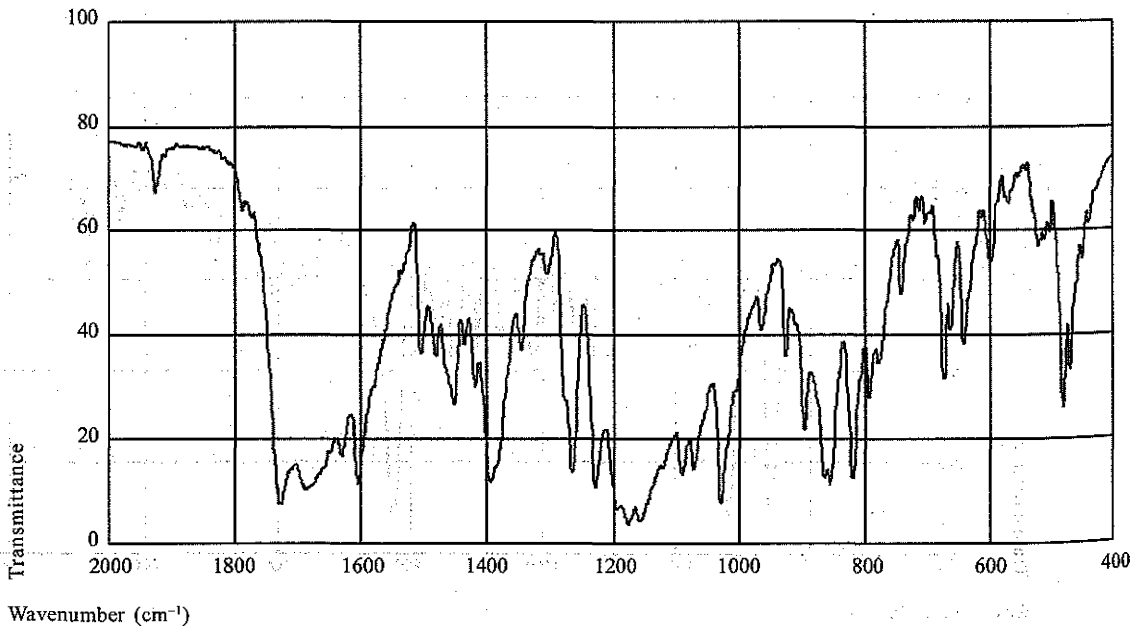
Nandrolone Laurate *Dispersive IR* Phase: KBr disc



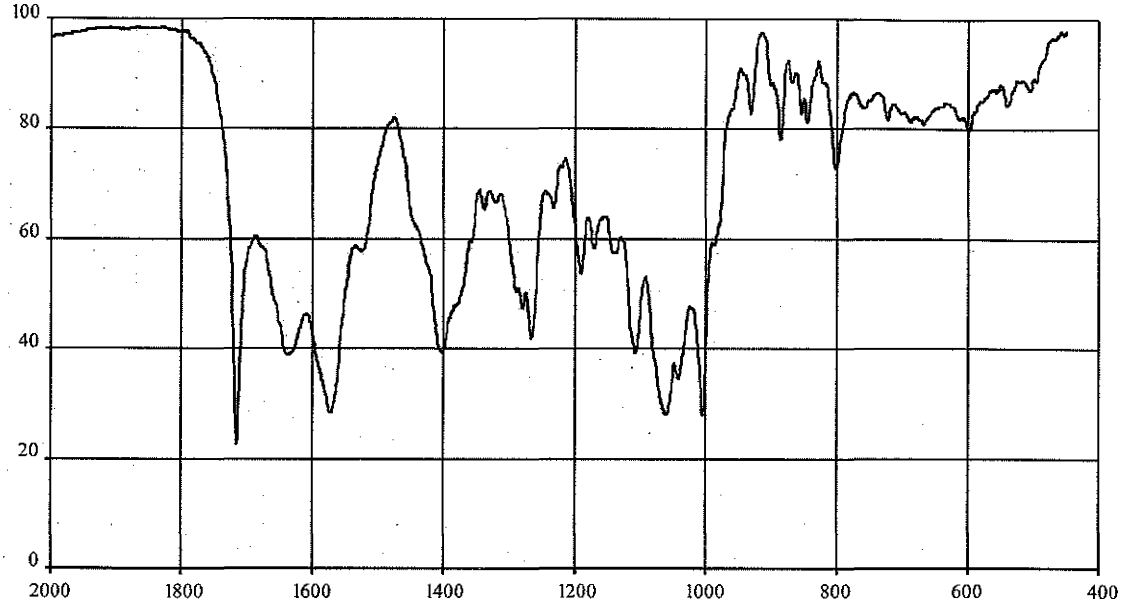
Nandrolone Phenylpropionate *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



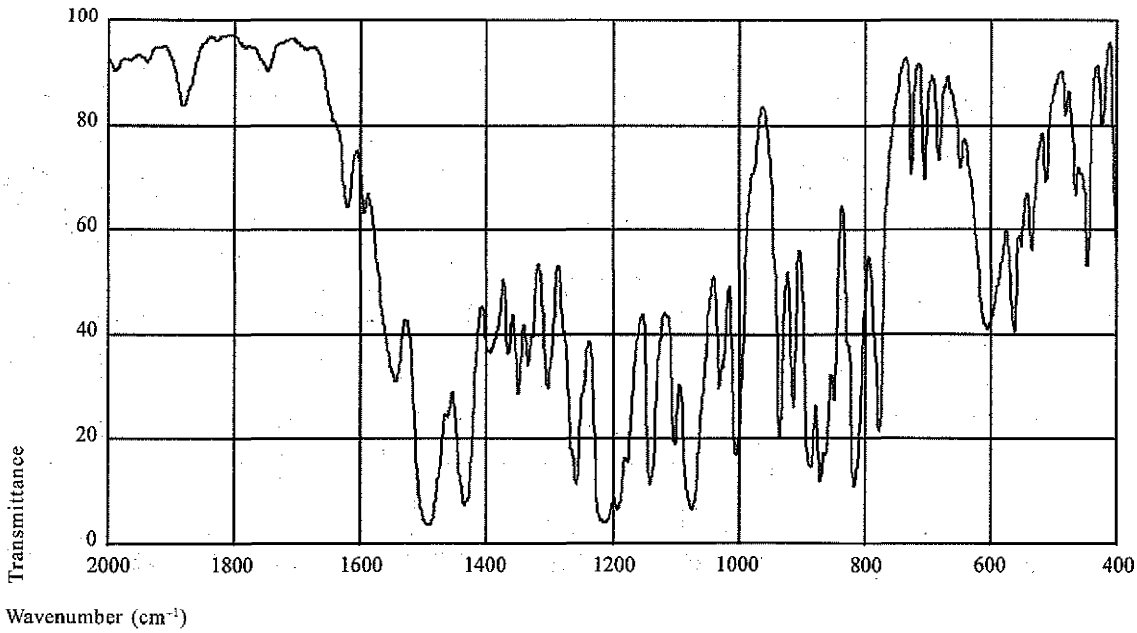
Naproxen *FTIR* Phase: KBr disc



Natamycin *FTIR* Phase: KBr disc



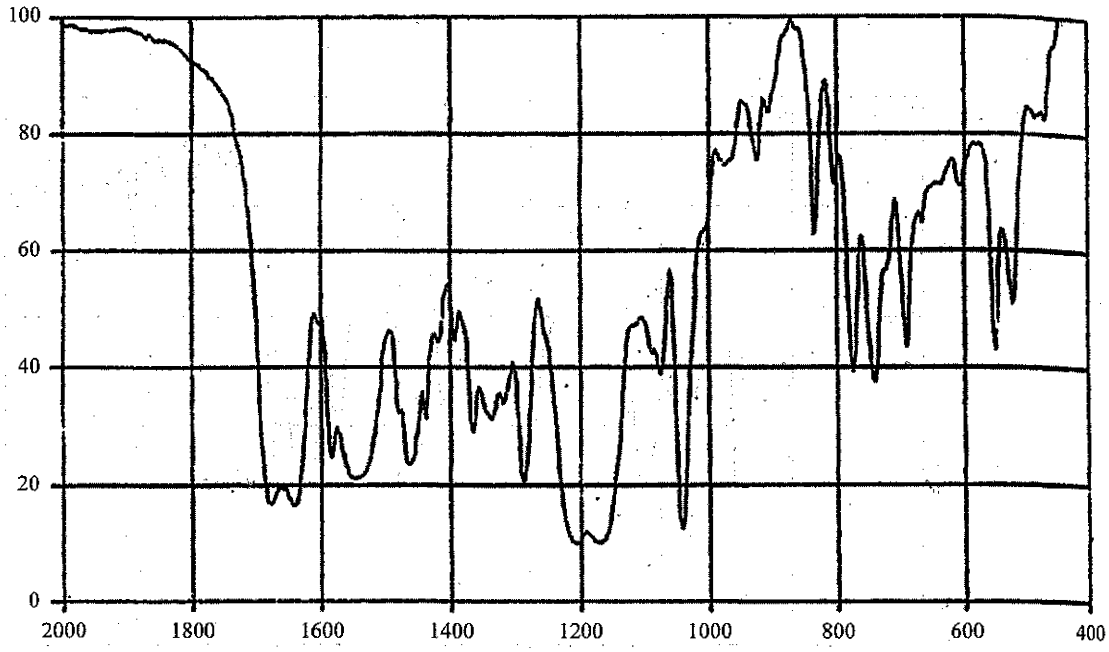
Nebivolol Hydrochloride *FTIR* Phase: KBr disc



Nelfinavir Mesylate

FTIR

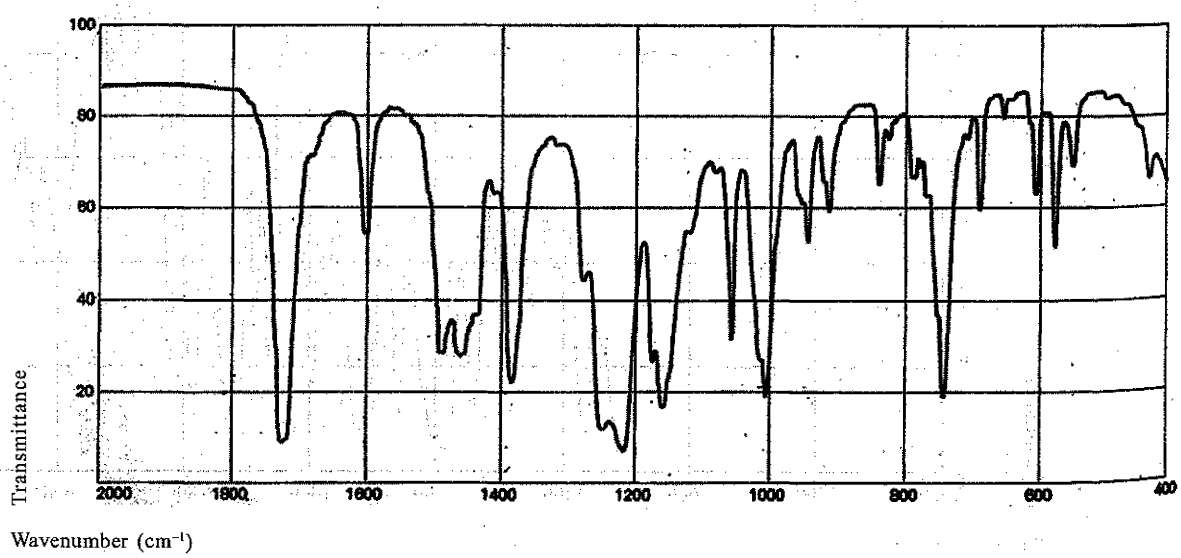
Phase: KBr disc



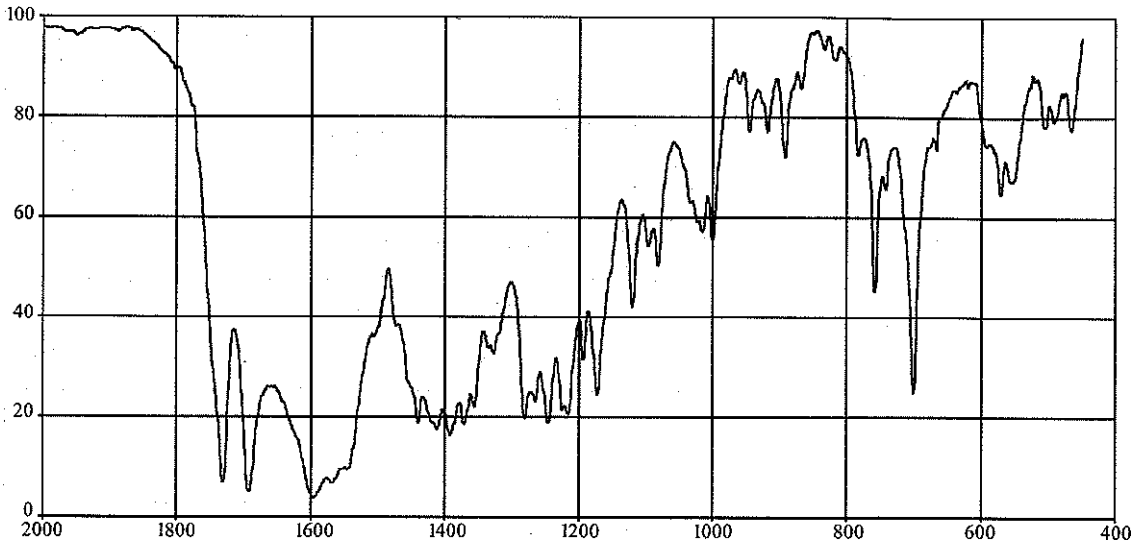
Neostigmine Methylsulphate

Dispersive IR

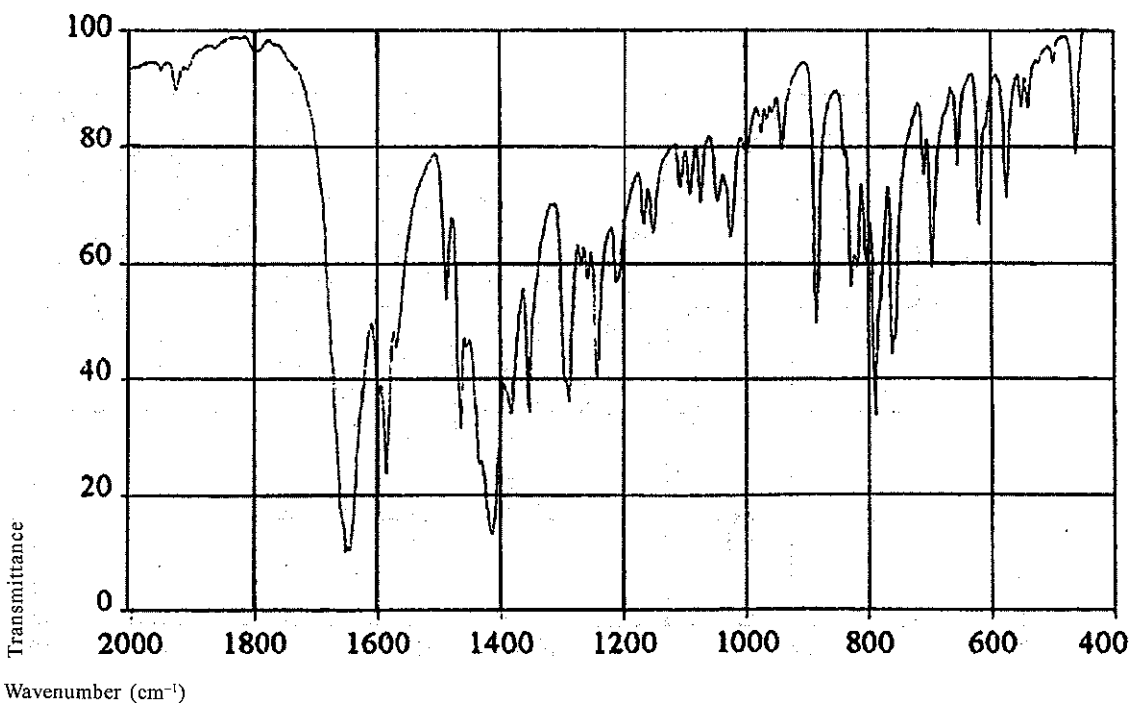
Phase: Liquid paraffin mull



Neotame *FTIR* Phase: KBr disc



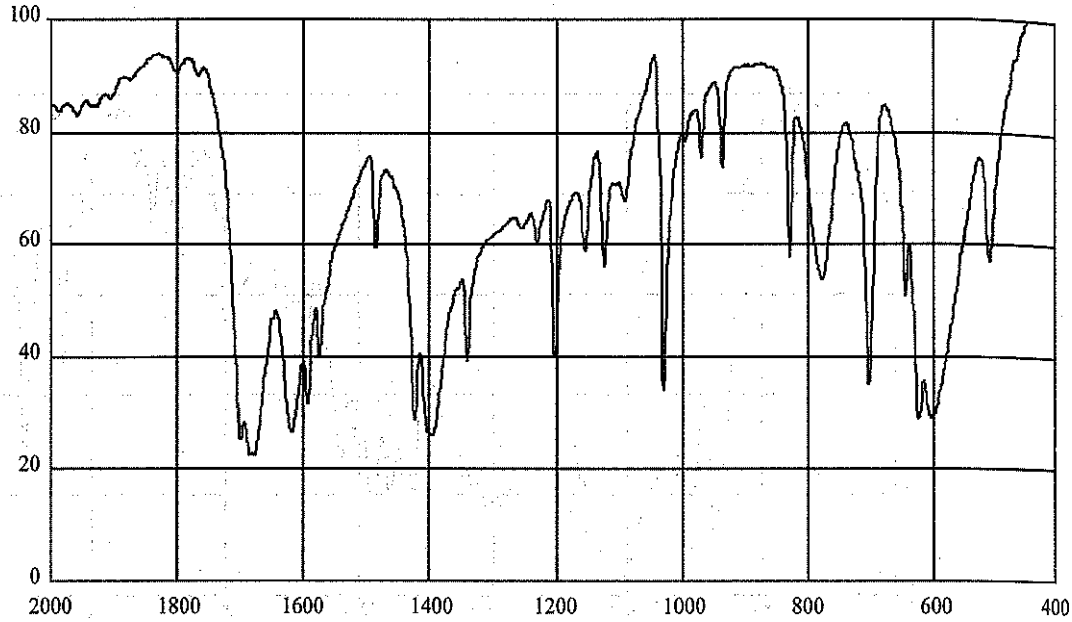
Nevirapine *FTIR* Phase: KBr disc



Niacinamide

FTIR

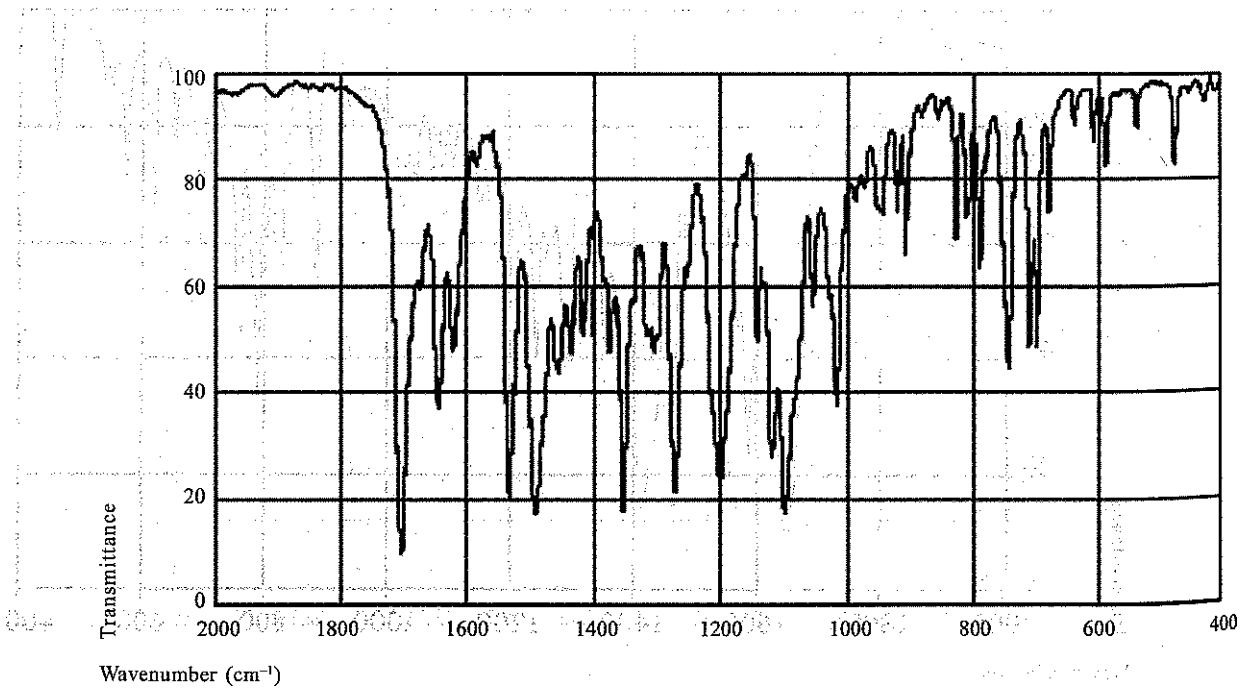
Phase: KBr disc



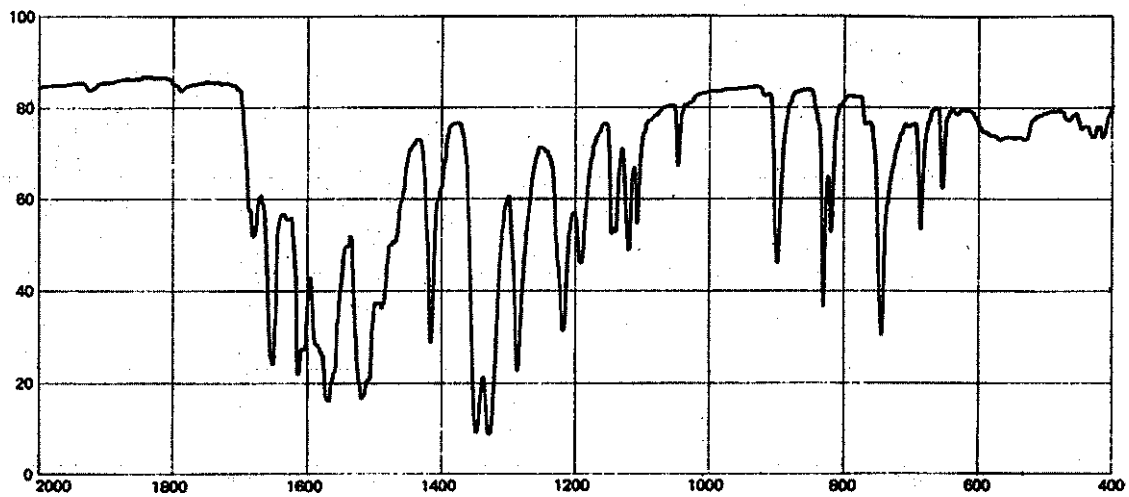
Nicardipine

FTIR

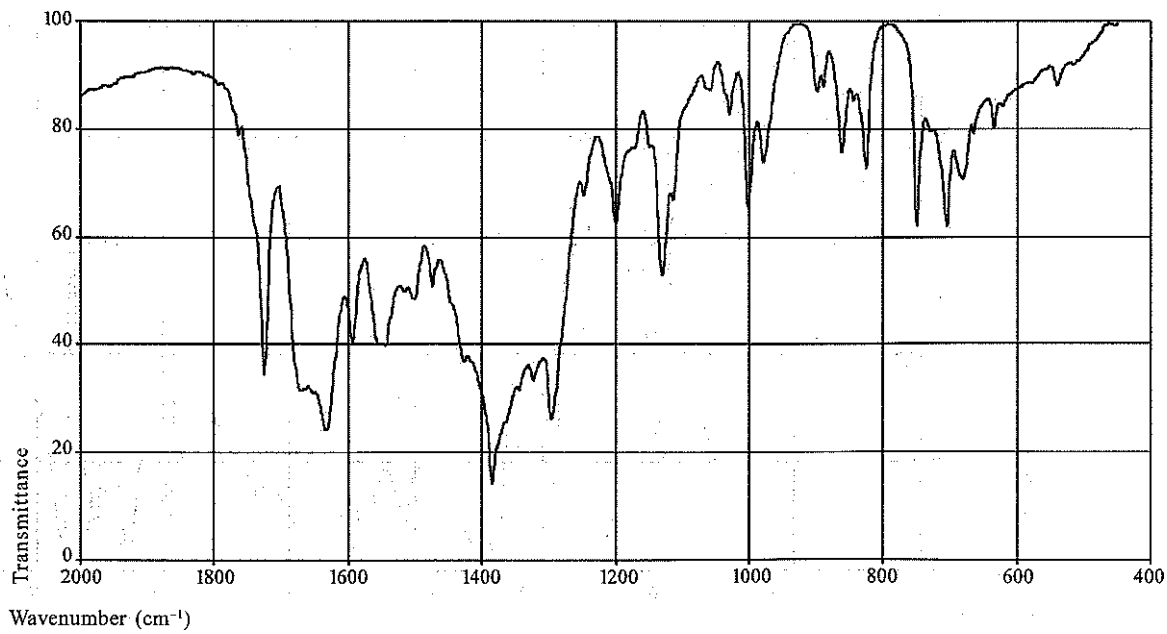
Phase: KBr disc



Niclosamide *Dispersive IR* Phase: KBr disc



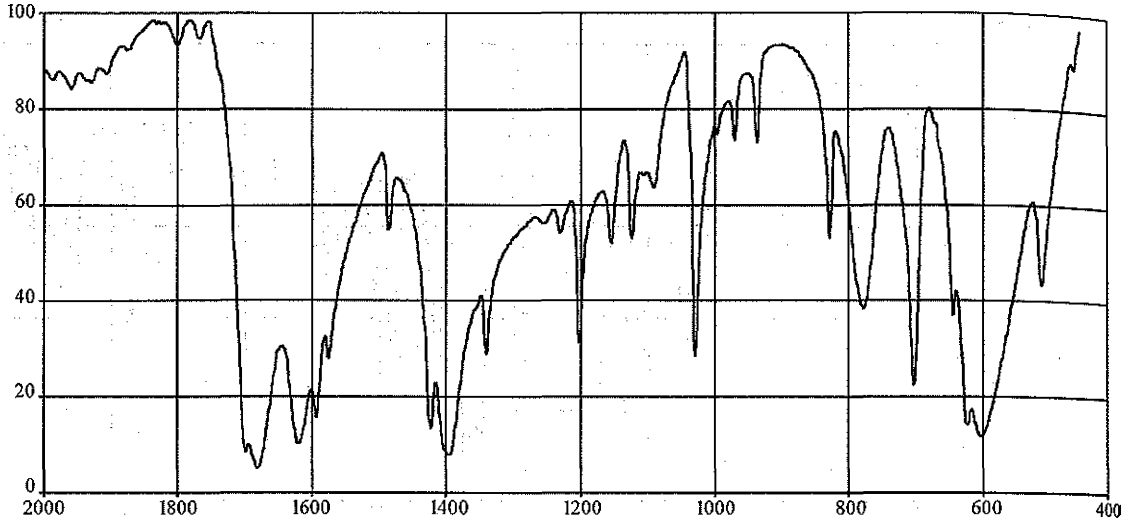
Nicorandil *FTIR* Phase: KBr disc



Nicotinamide

FTIR

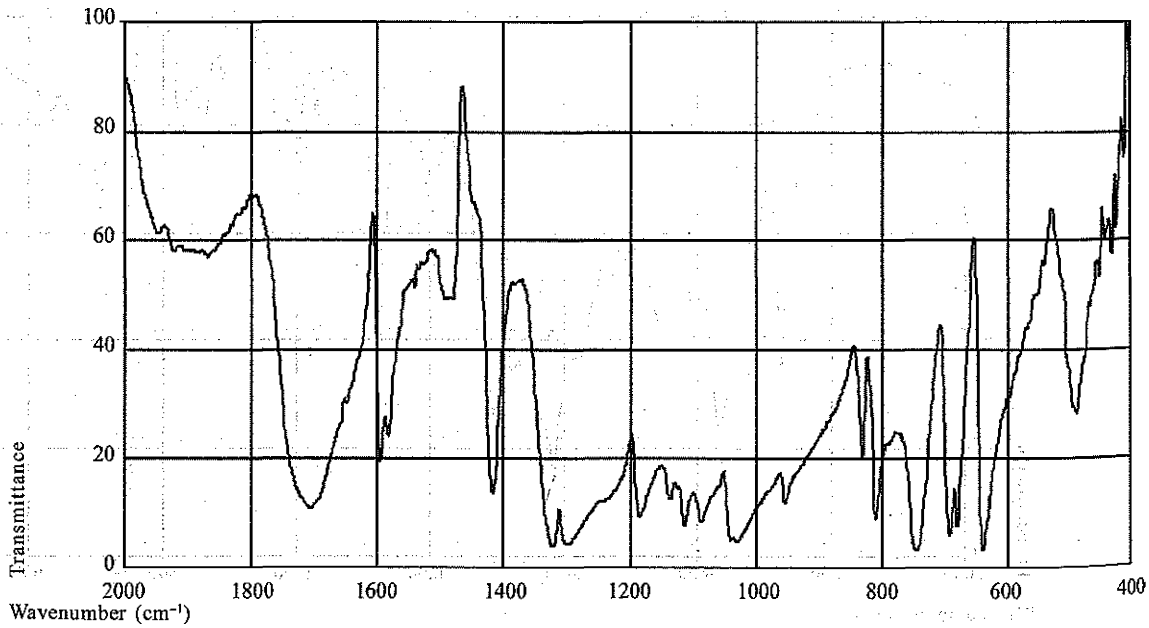
Phase: KBr disc



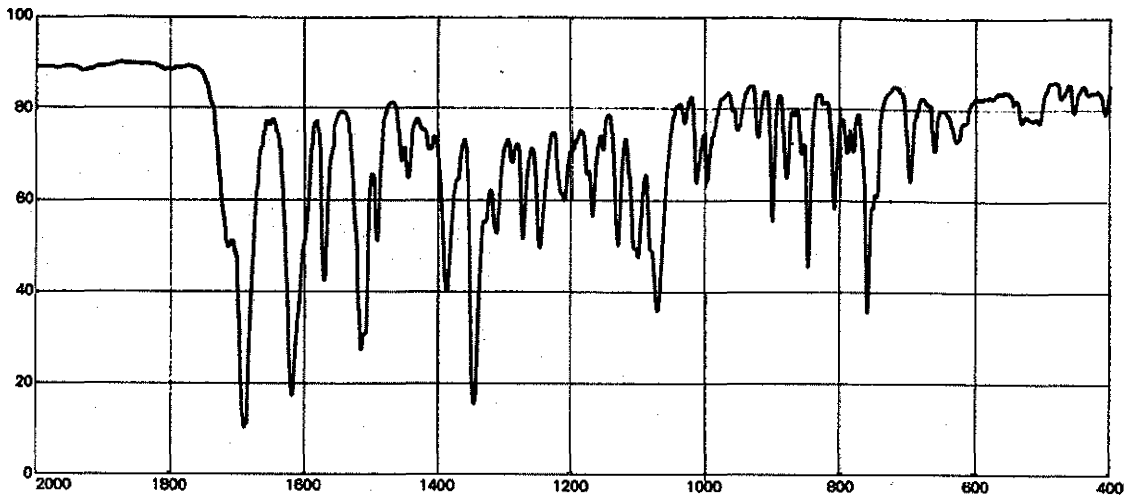
Nicotinic Acid

FTIR

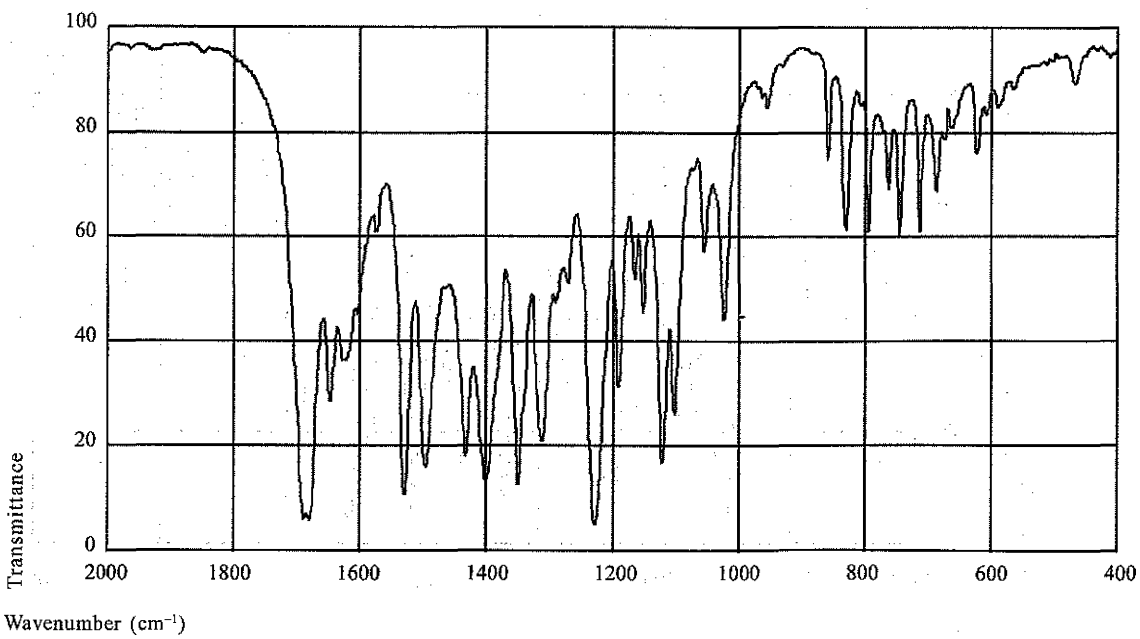
Phase: KBr disc



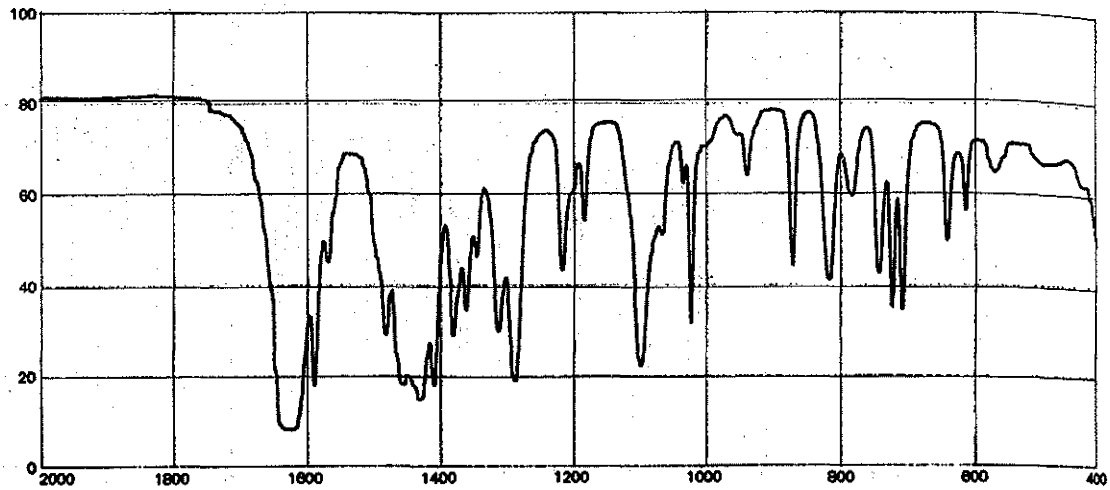
Nicoumalone *Dispersive IR* Phase: KBr disc



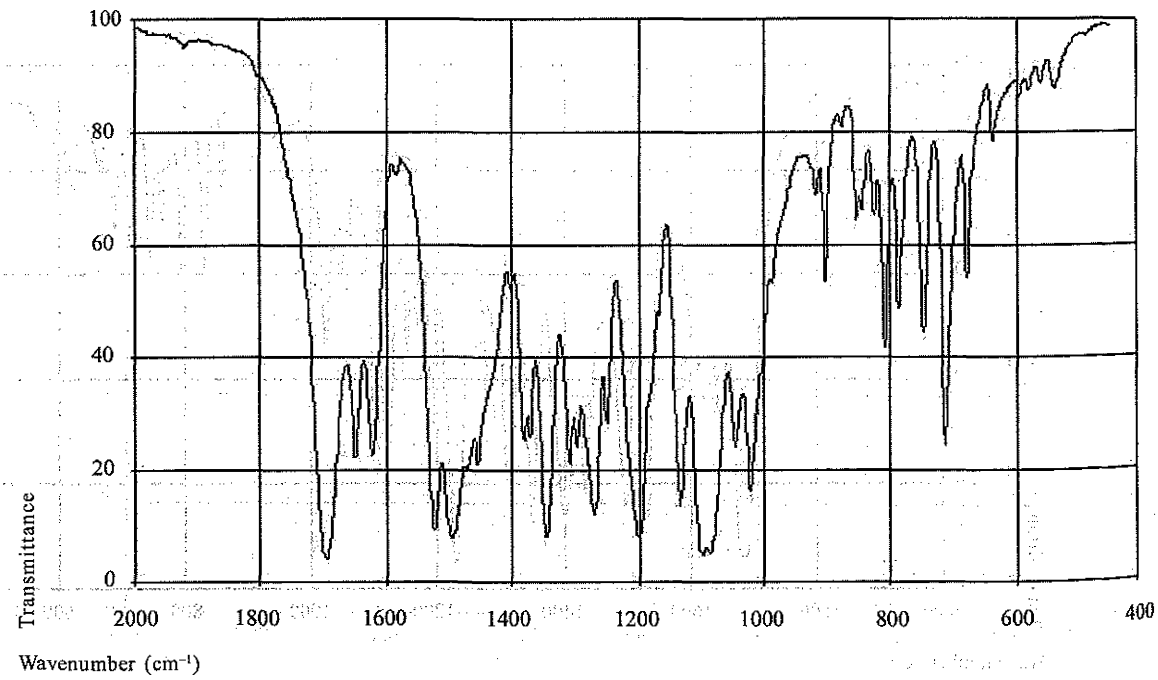
Nifedipine *FTIR* Phase: KBr disc



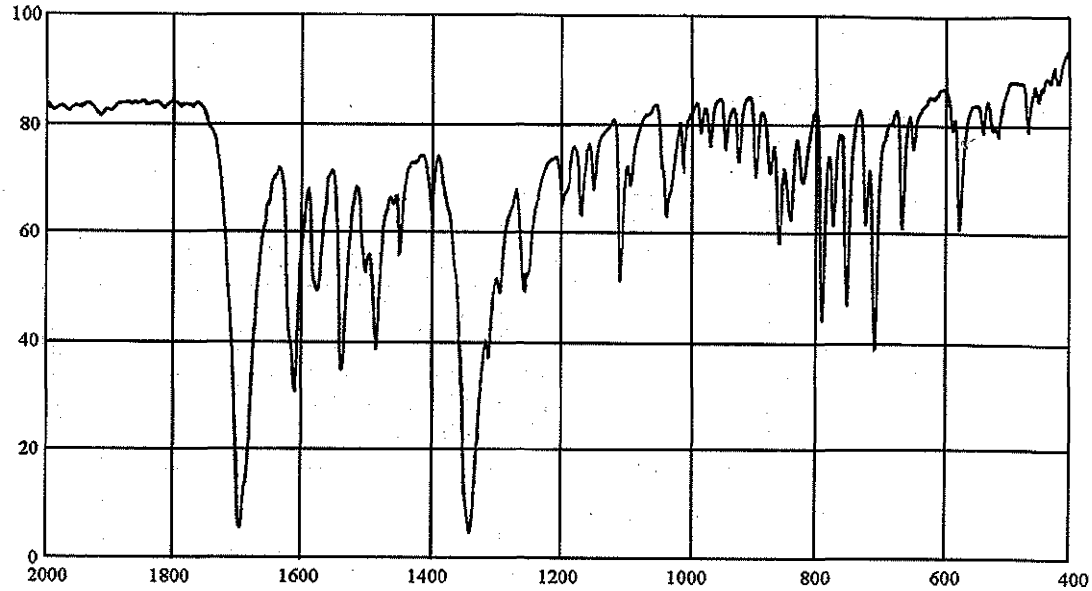
Nikethamide *Dispersive IR* Phase: Thin film



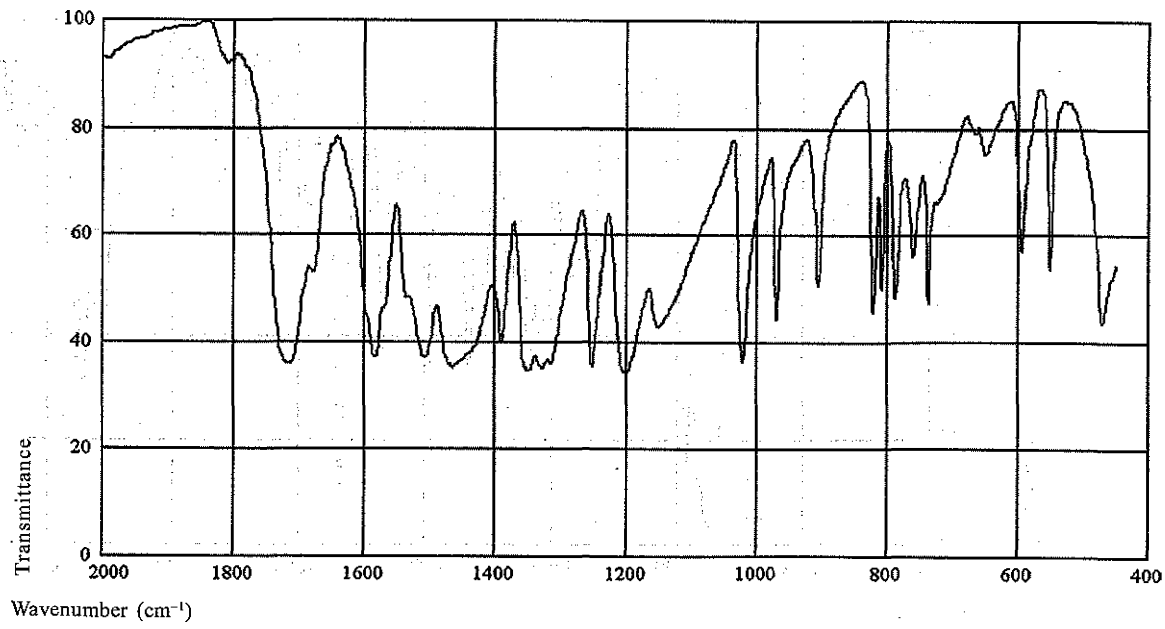
Nimodipine *FTIR* Phase: KBr disc



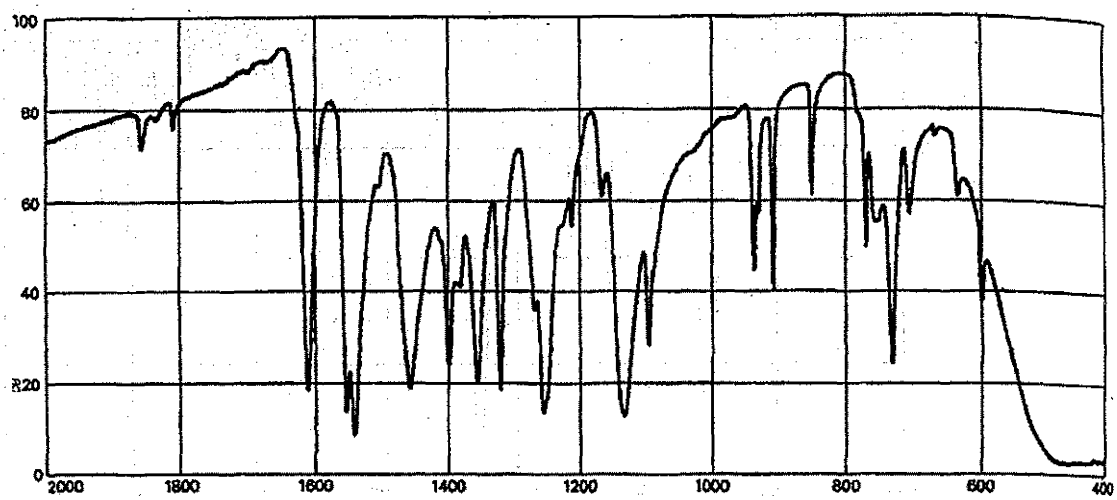
Nitrazepam *FTIR* Phase: KBr disc



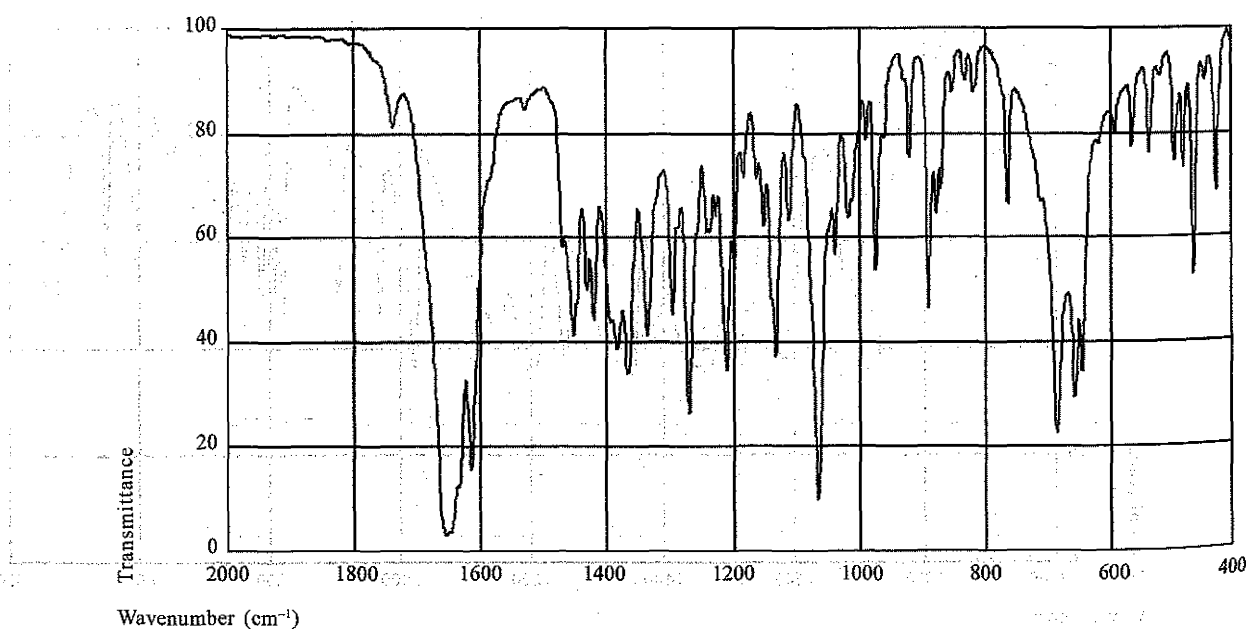
Nitrofurazone *FTIR* Phase: KBr disc



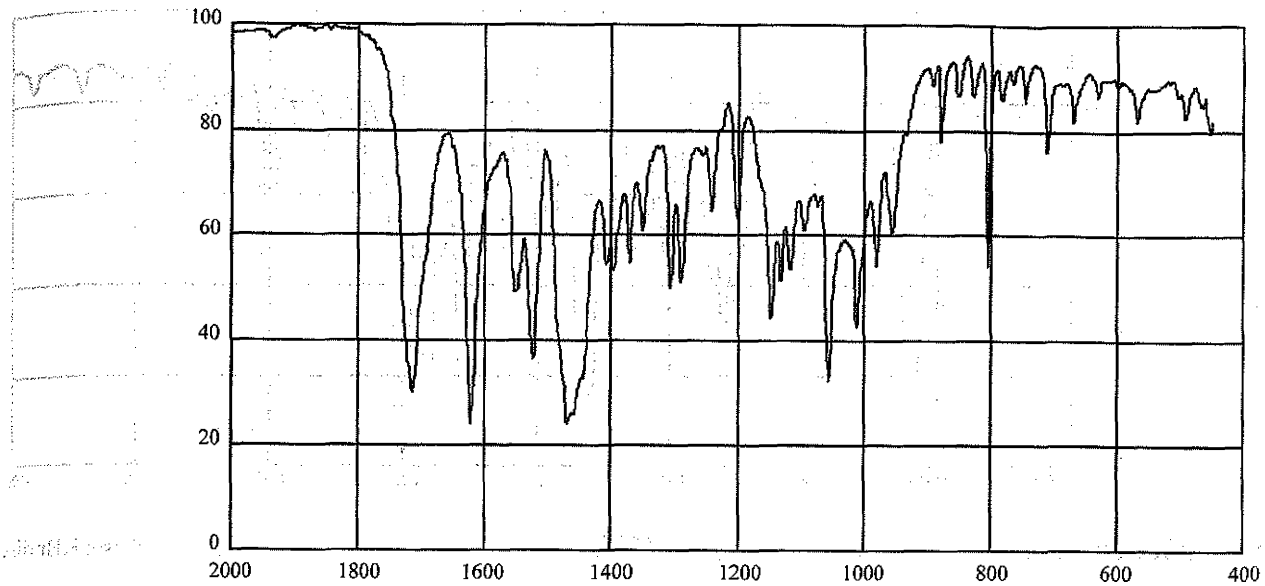
Nitroxynil *Dispersive IR* Phase: Liquid paraffin mull



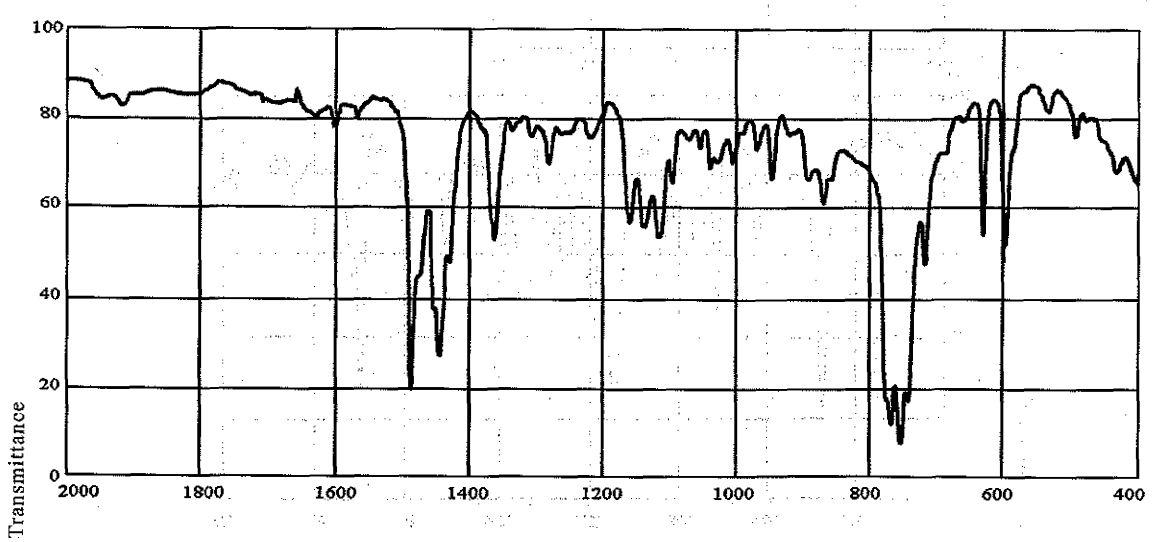
Norethisterone *FTIR* Phase: KBr disc



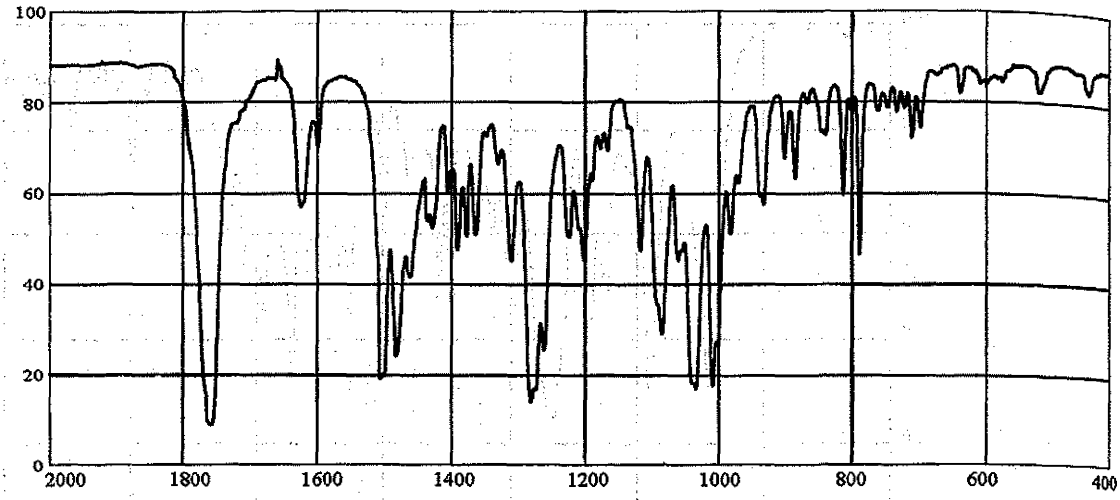
Norfloxacin *FTIR* Phase: KBr disc



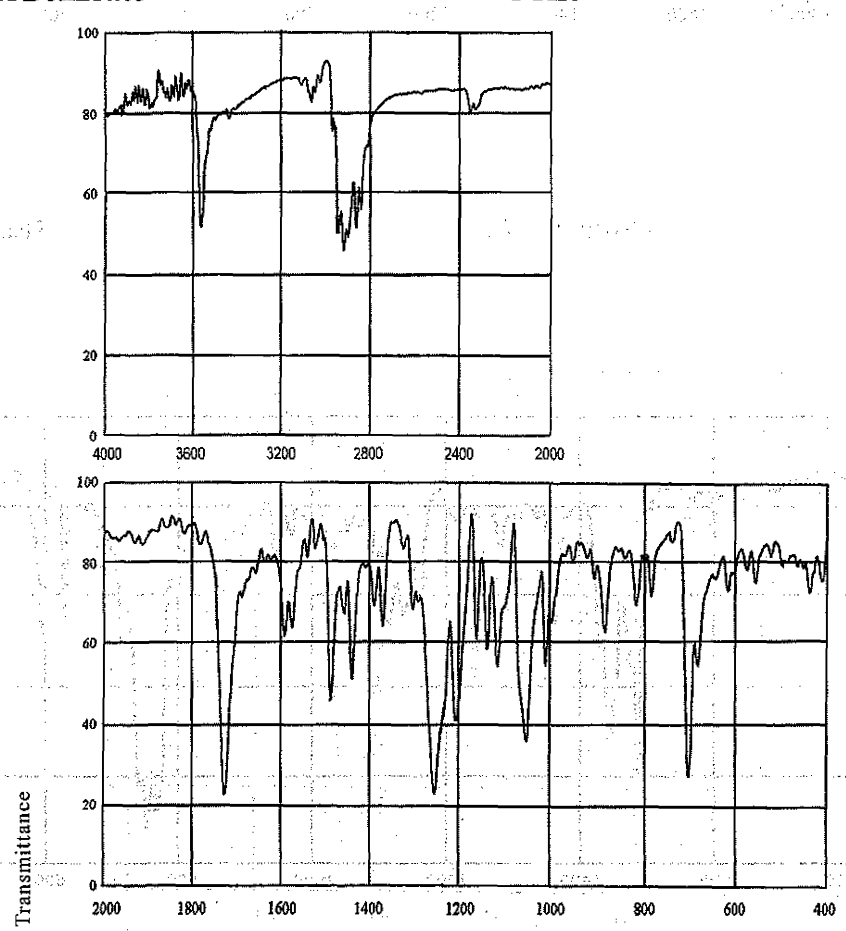
Nortriptyline *Dispersive IR* Phase: Thin film



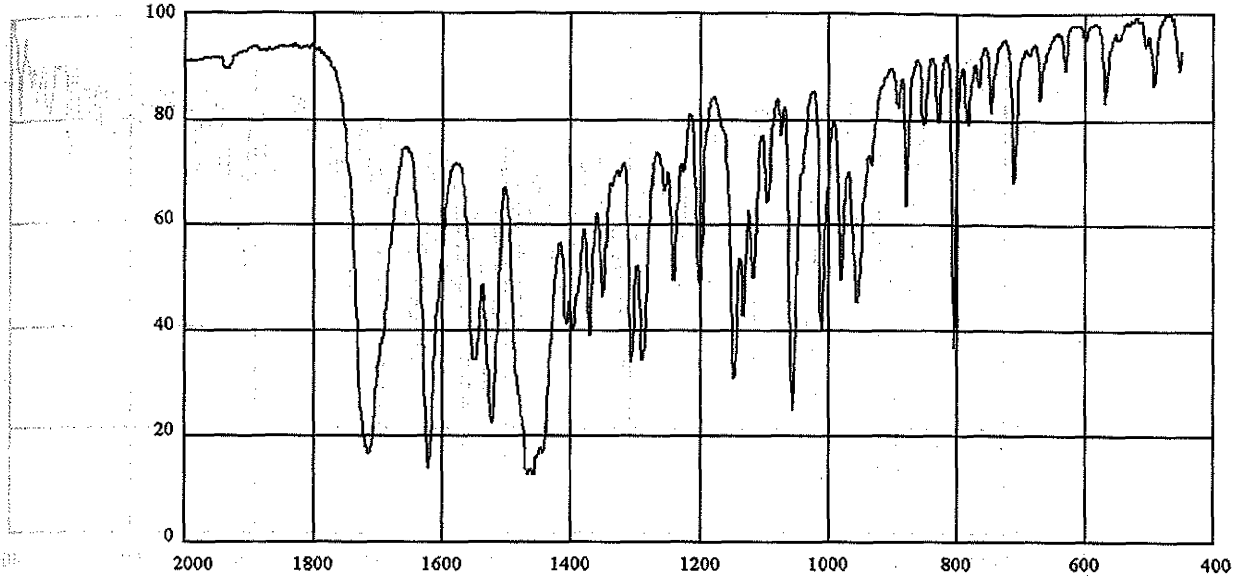
Noscapine *Dispersive IR* Phase: KBr disc



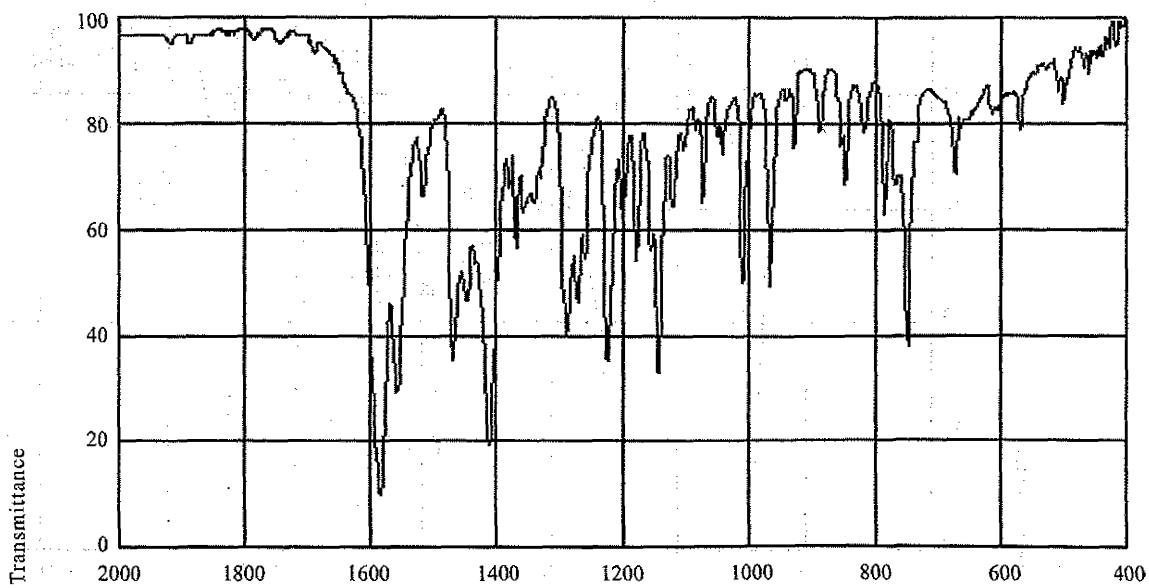
Oestradiol Benzoate *FTIR* Phase: KBr disc



Ofloxacin *FTIR* Phase: KBr disc



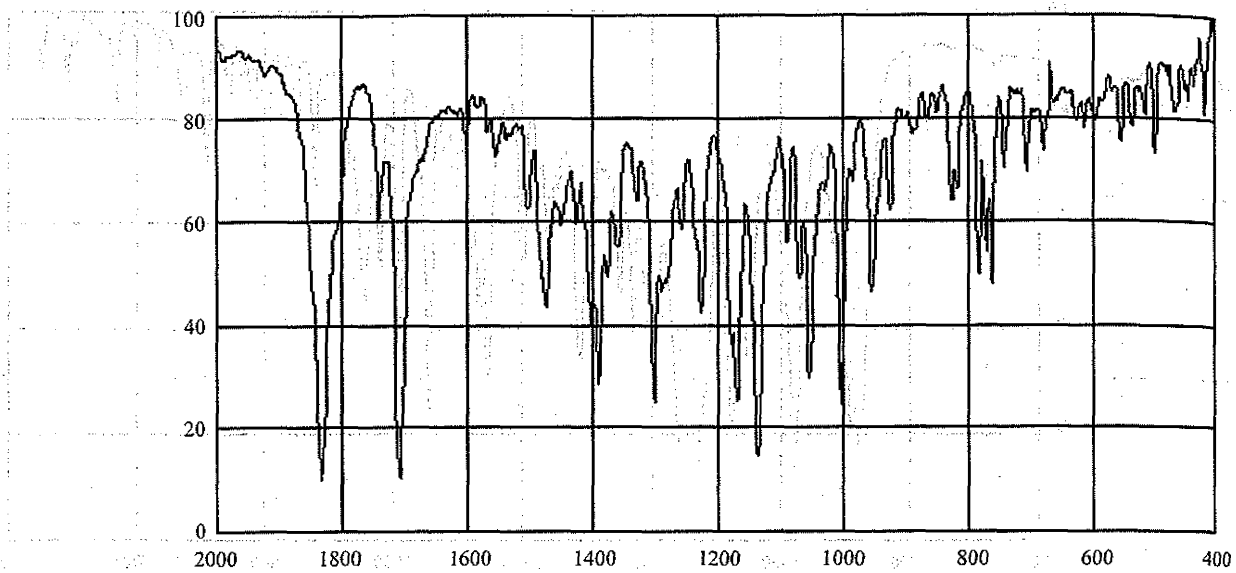
Olanzapine *FTIR* Phase: KBr disc



Olmesartan Medoxomil

FTIR

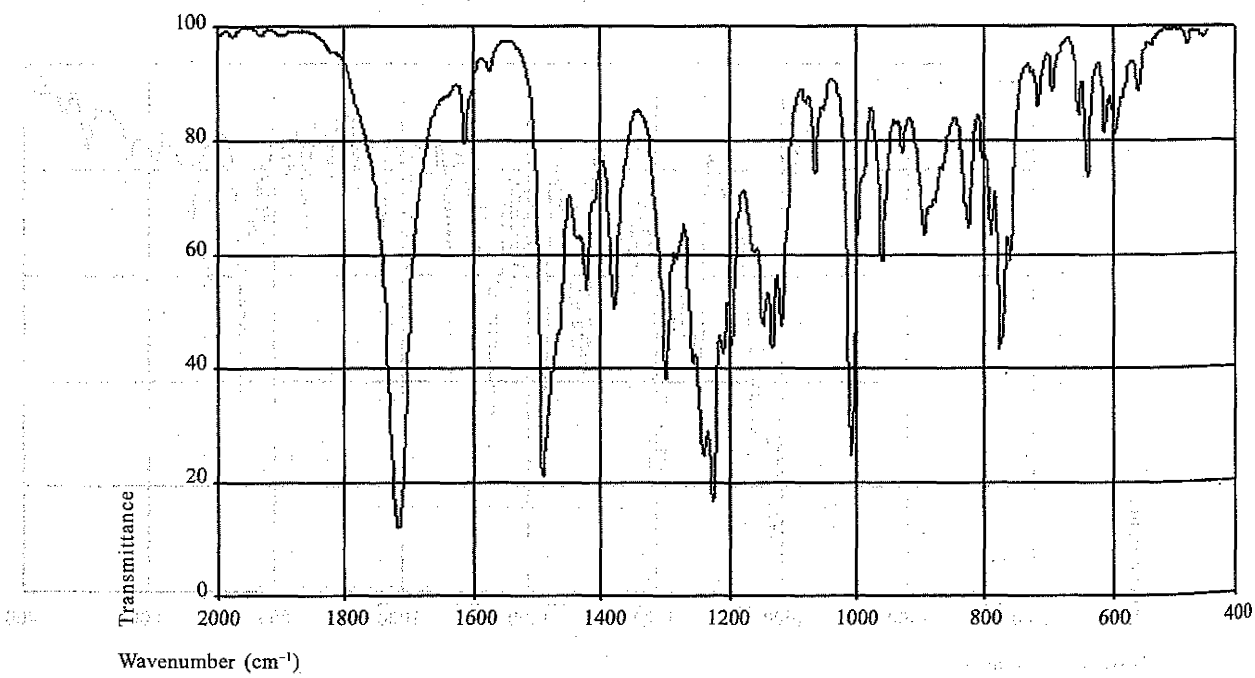
Phase: KBr disc



Olopatadine Hydrochloride

FTIR

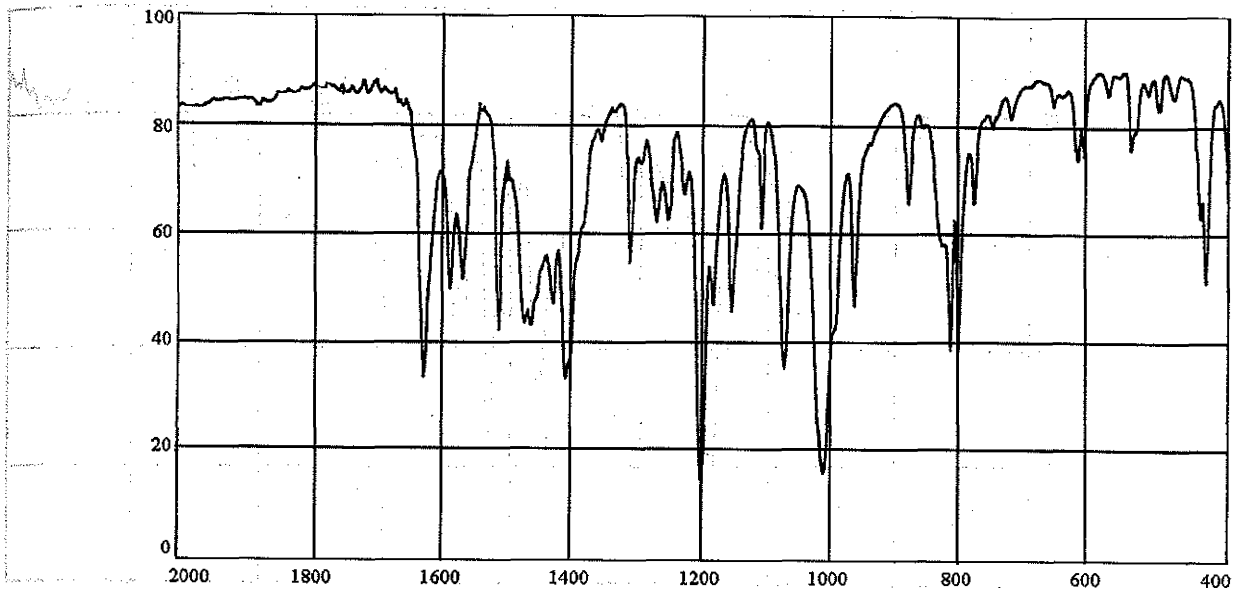
Phase: KBr disc



Omeprazole

FTIR

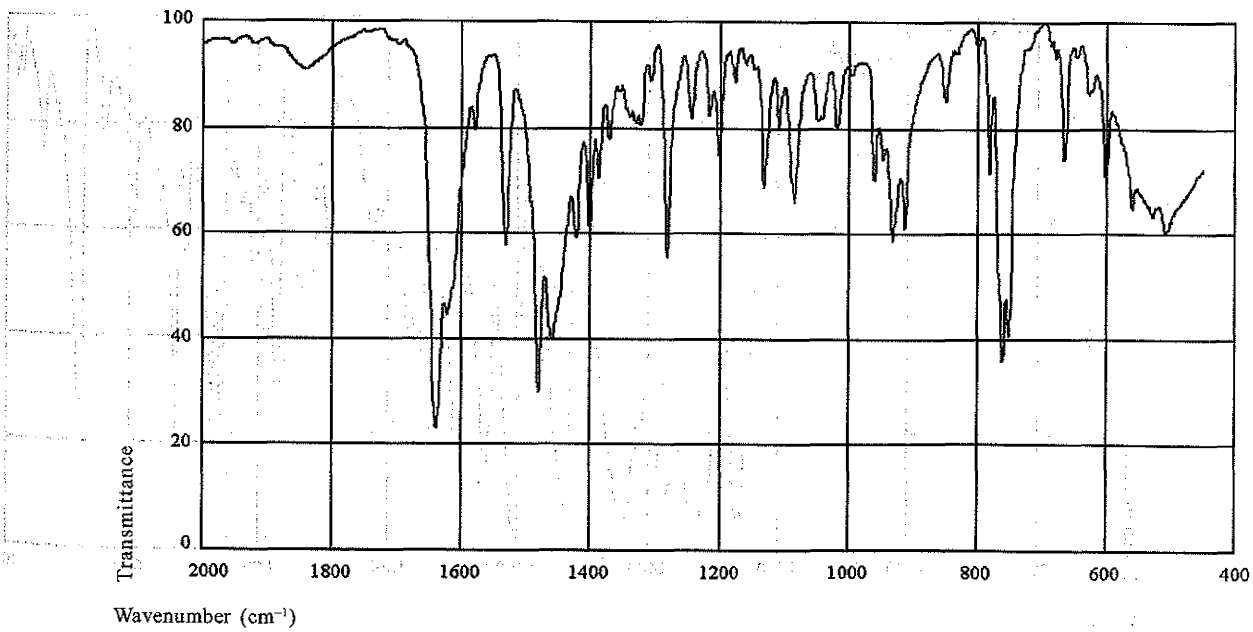
Phase: KBr disc



Ondansetron Hydrochloride

FTIR

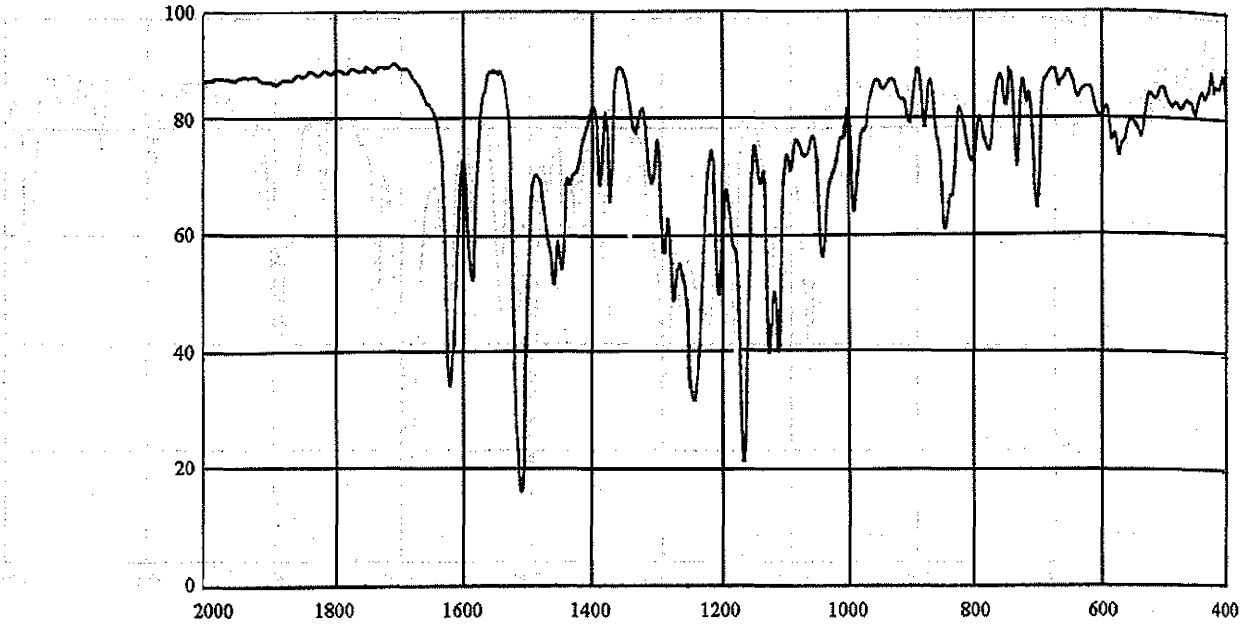
Phase: KBr disc



Ormeloxifene Hydrochloride

FTIR

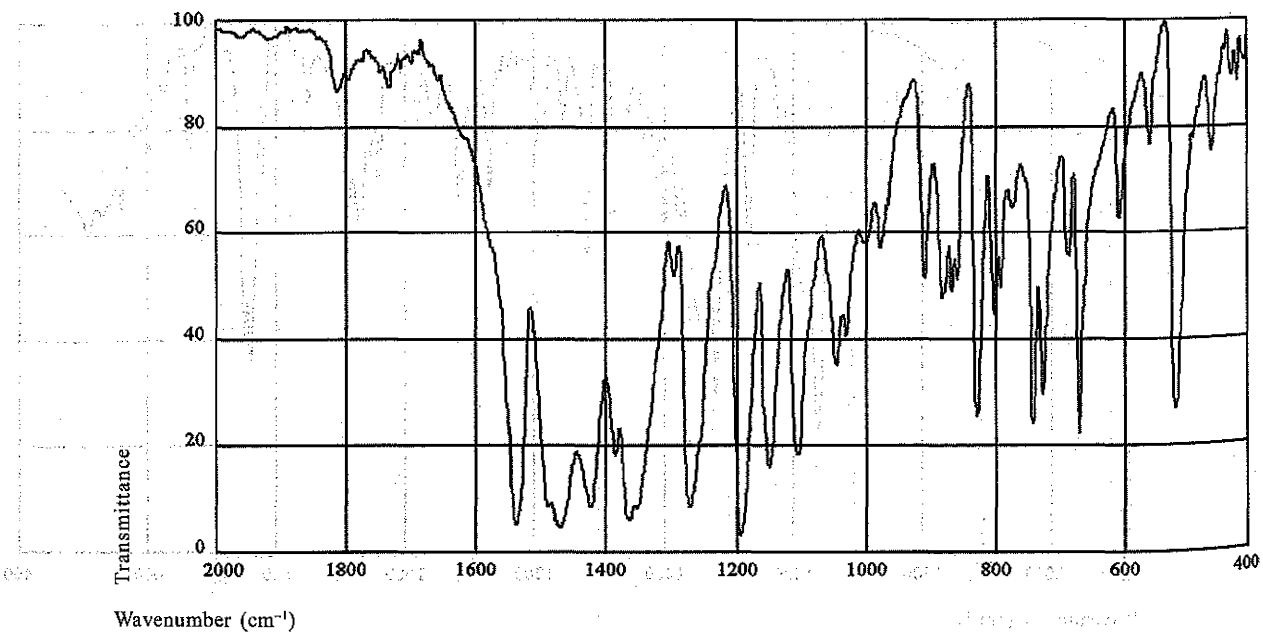
Phase: KBr disc



Ornidazole

FTIR

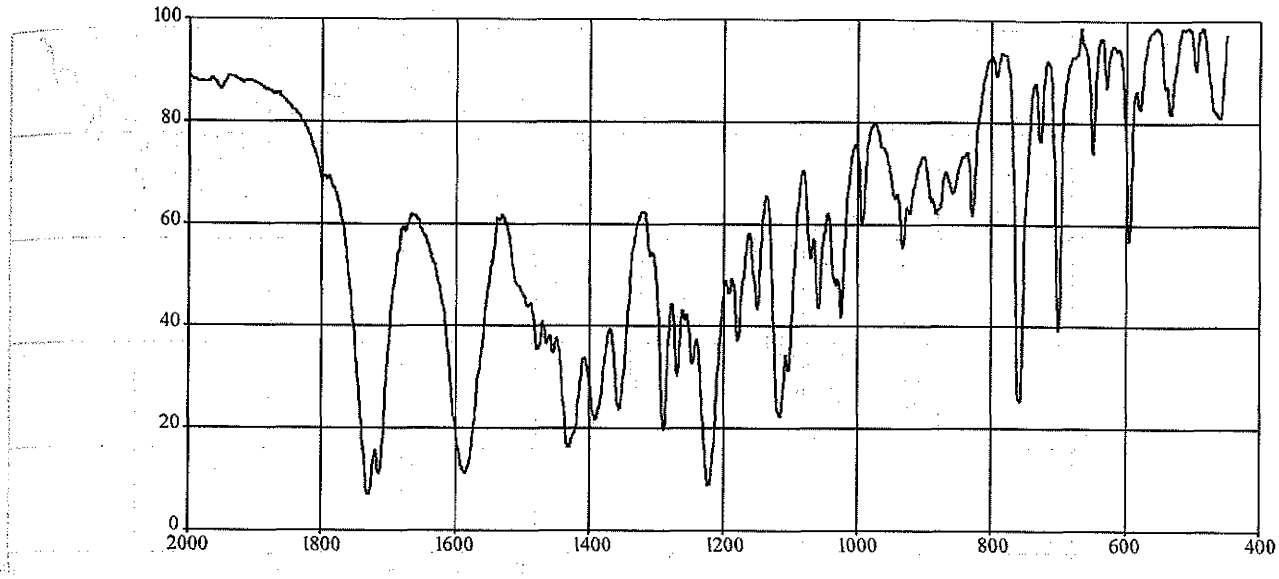
Phase: KBr disc



Orphenadrine Citrate

FTIR

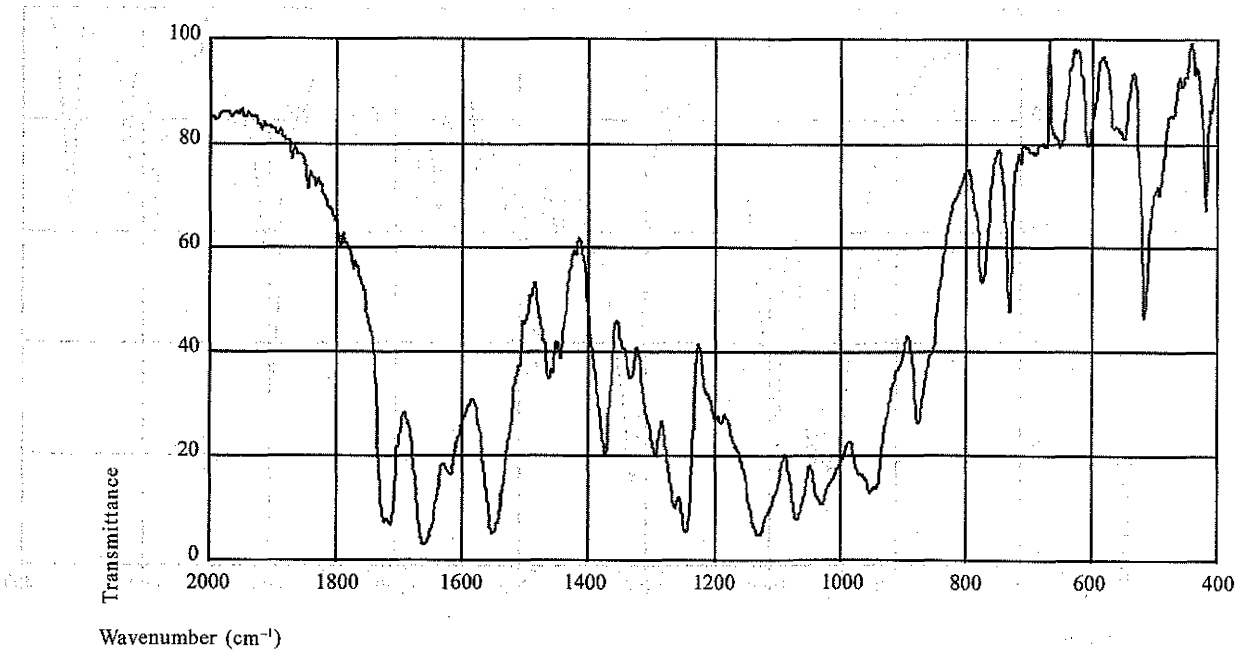
Phase: KBr disc



Oseltamivir Phosphate

FTIR

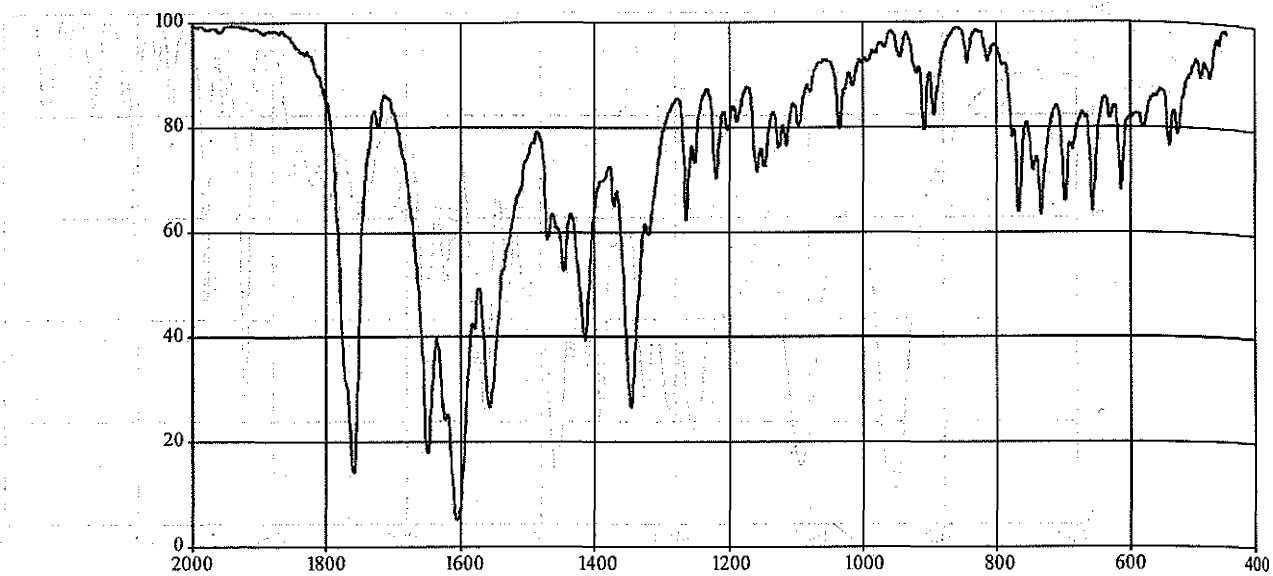
Phase: KBr disc



Oxacillin Sodium Monohydrate

FTIR

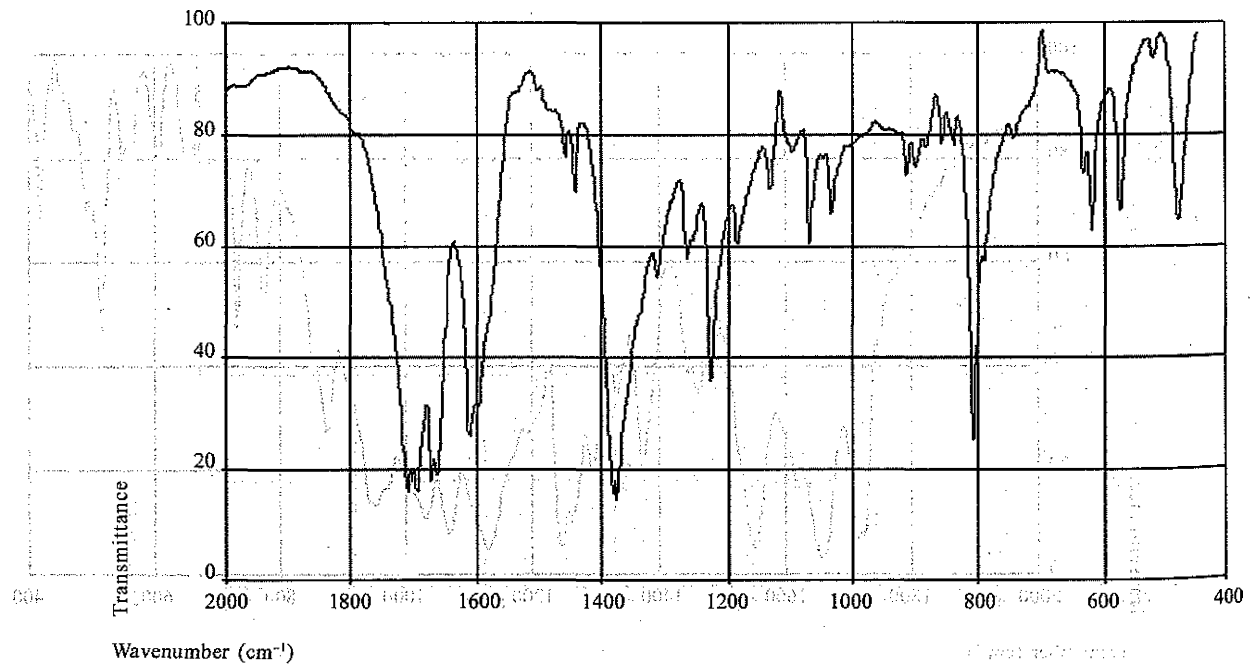
Phase: KBr disc



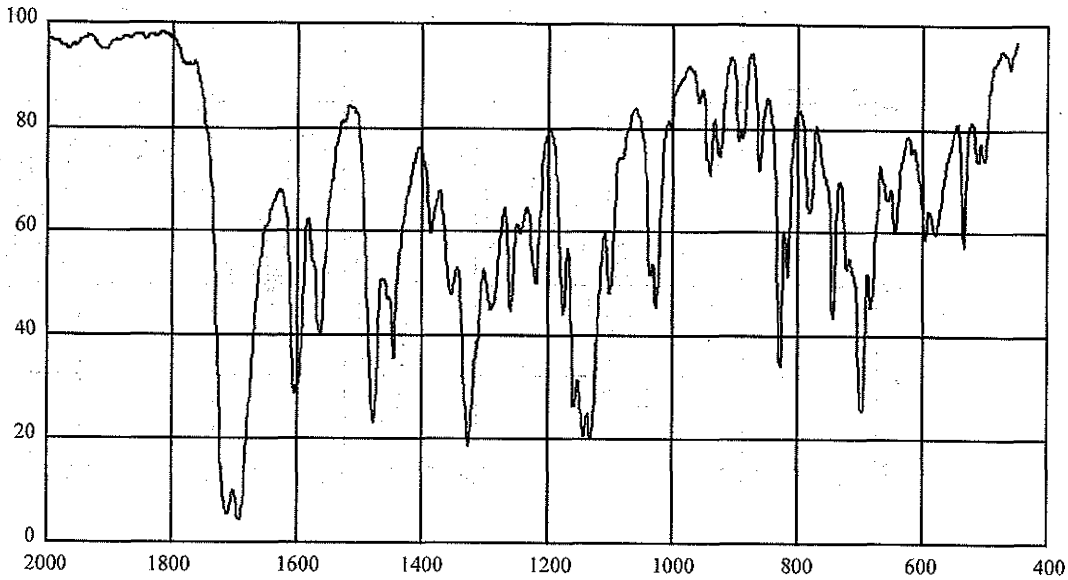
Oxaliplatin

FTIR

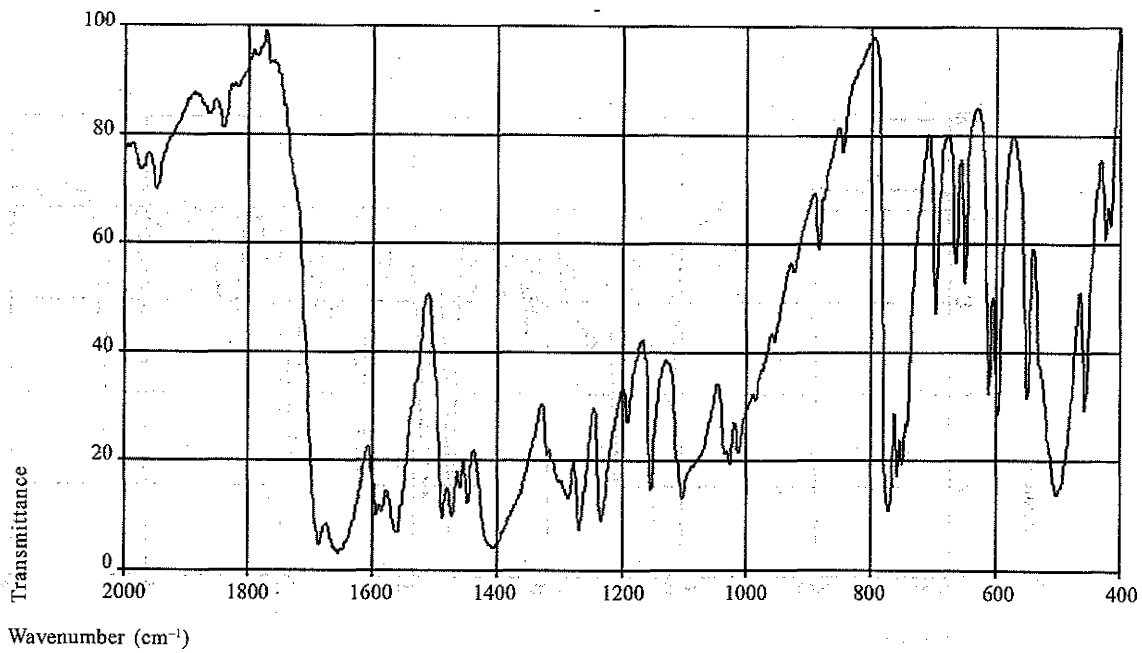
Phase: KBr disc



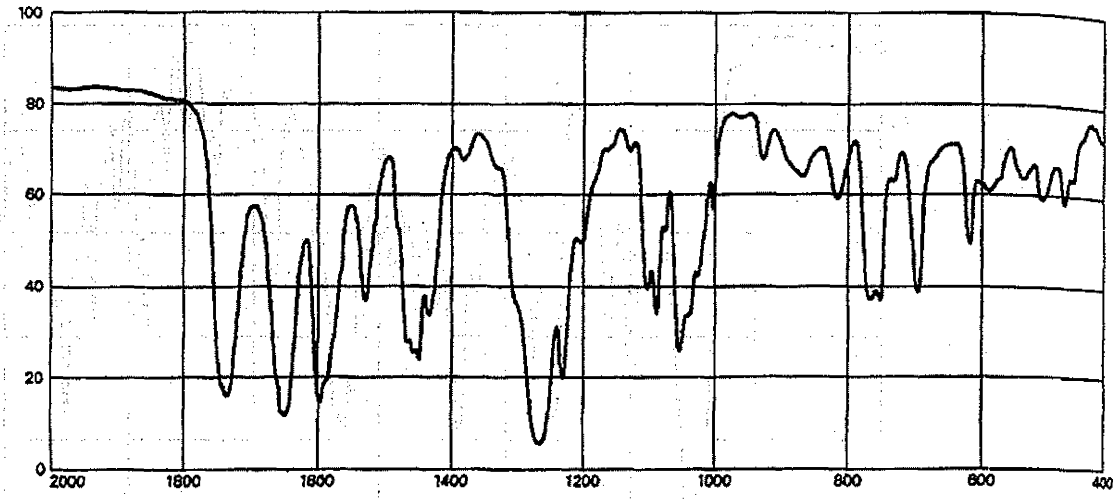
Oxazepam *FTIR* Phase: KBr disc



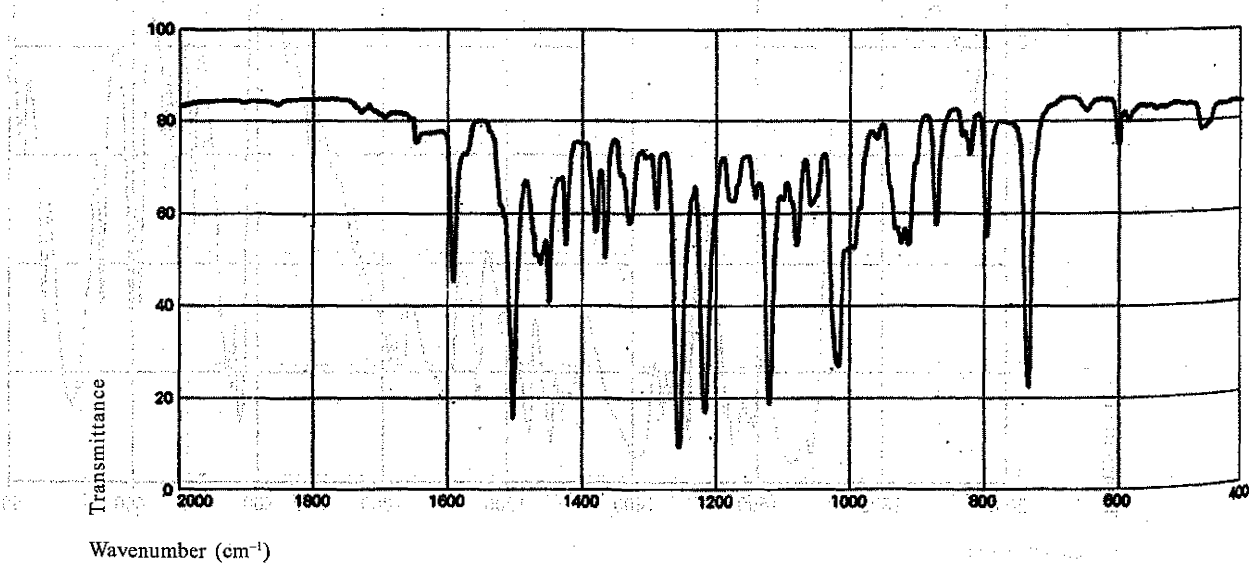
Oxcarbazepine *FTIR* Phase: KBr disc



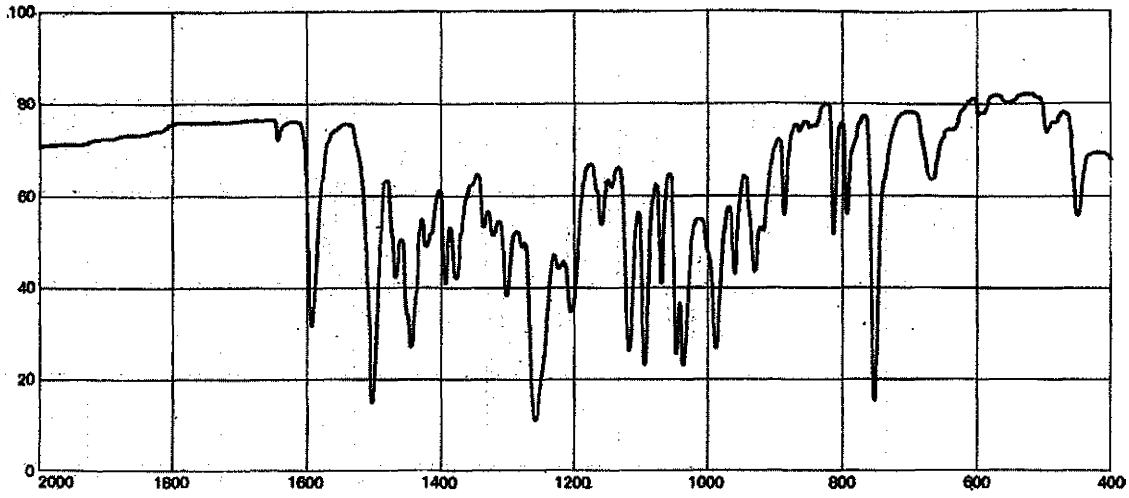
Oxfendazole *Dispersive IR* Phase: KBr disc



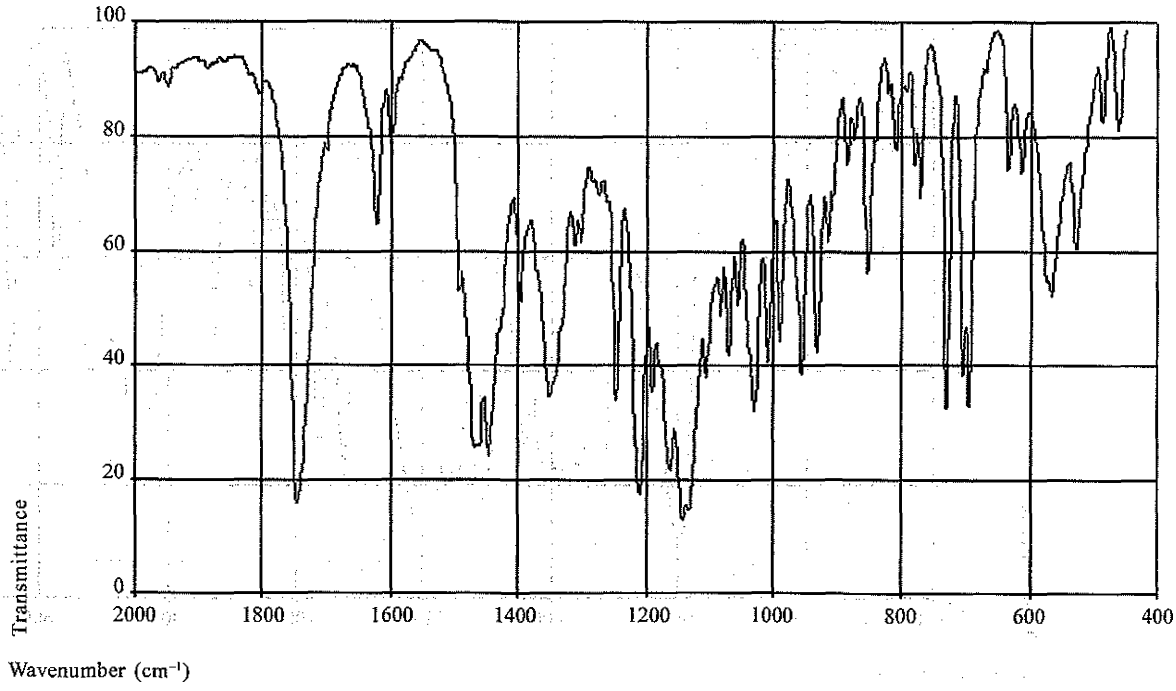
Oxprenolol *Dispersive IR* Phase: KBr disc



Oxprenolol Hydrochloride *Dispersive IR* Phase: KCl disc

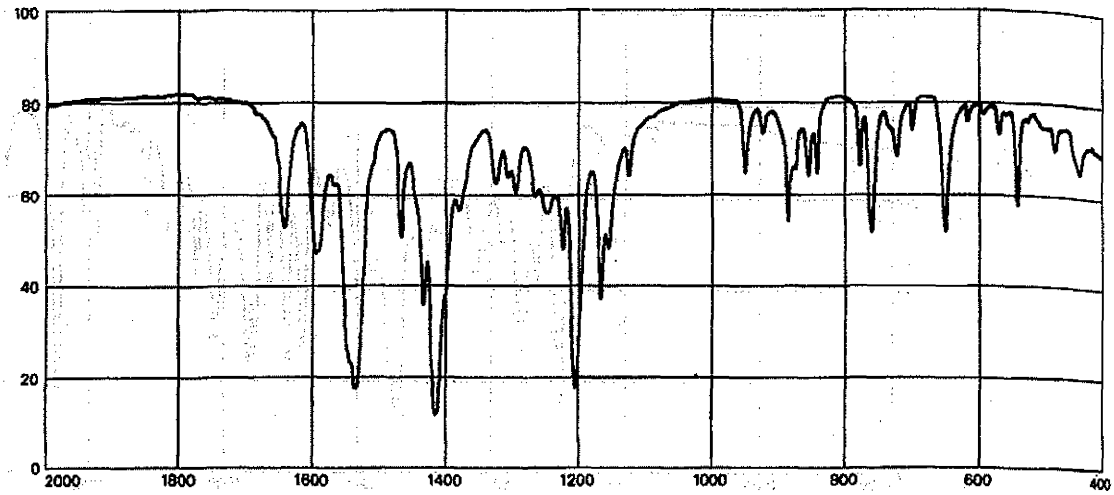


Oxybutynin Hydrochloride *FTIR* Phase: KBr disc



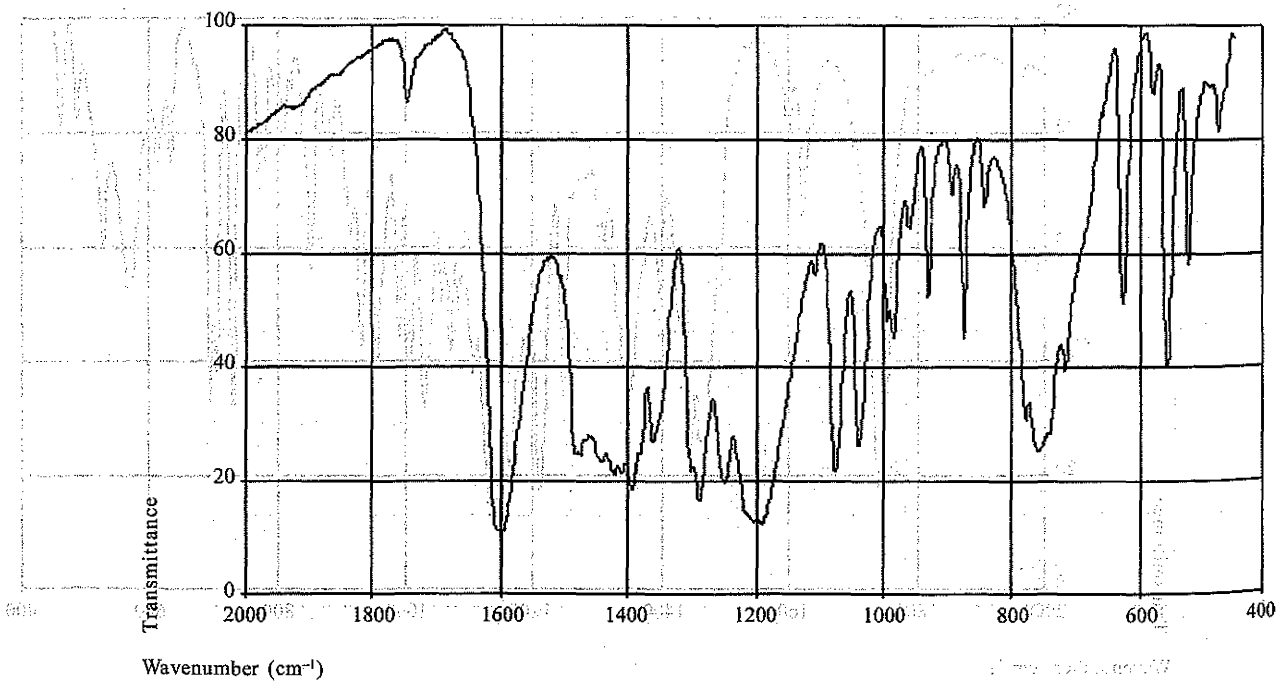
Oxyclozanide

Dispersive IR Phase: KBr disc

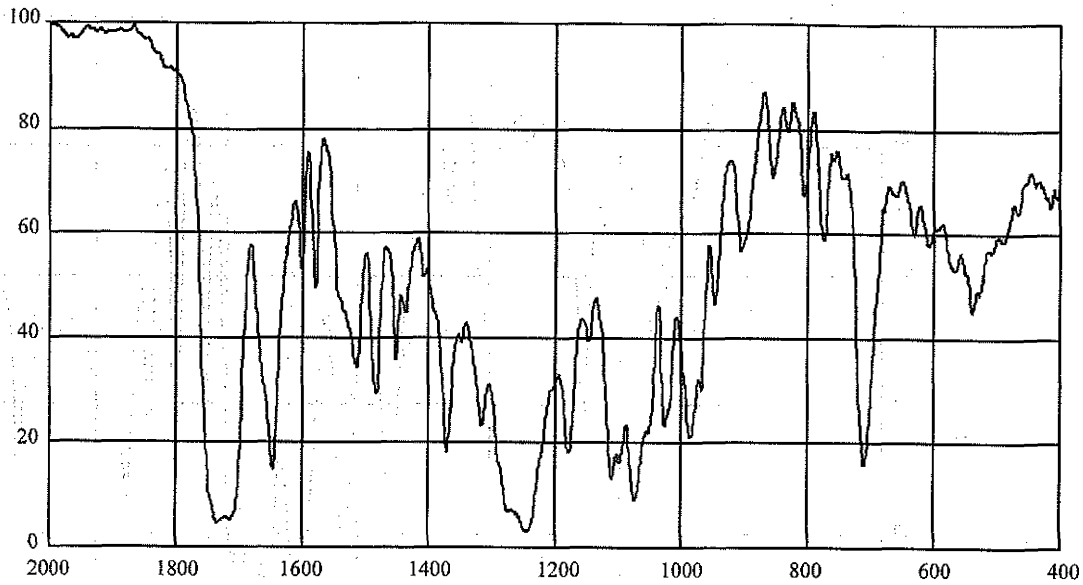


Oxymetazoline Hydrochloride

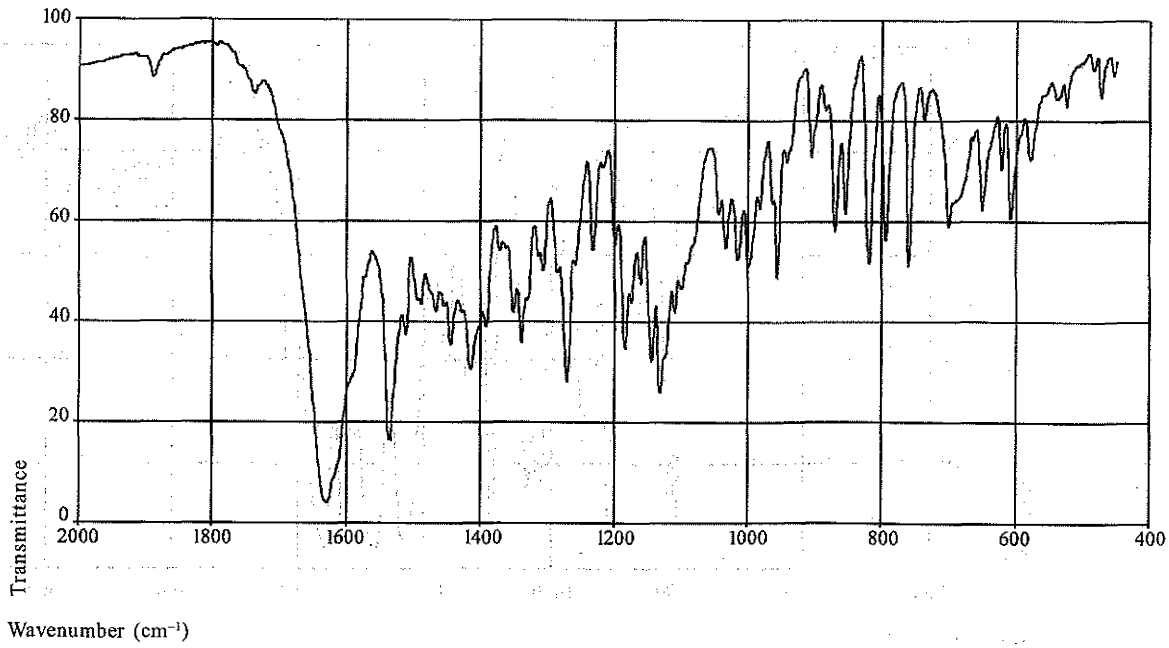
FTIR Phase: KBr disc



Paclitaxel *FTIR* Phase: KBr disc



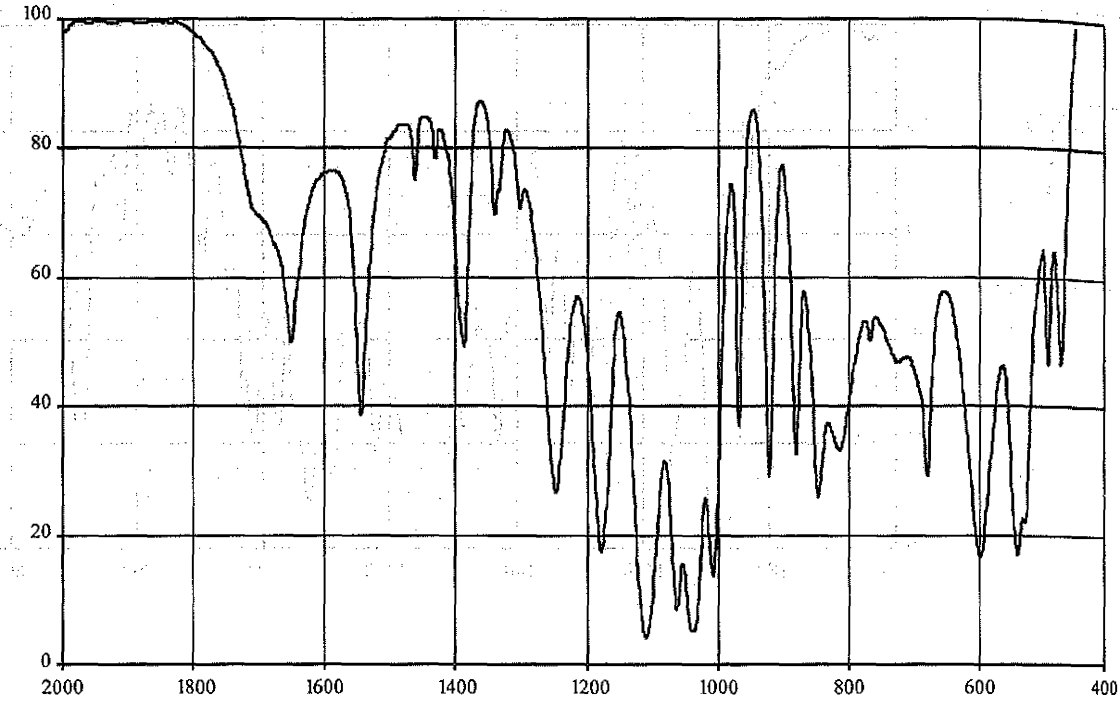
Paliperidone *FTIR* Phase: KBr disc



Pamidronate Disodium

FTIR

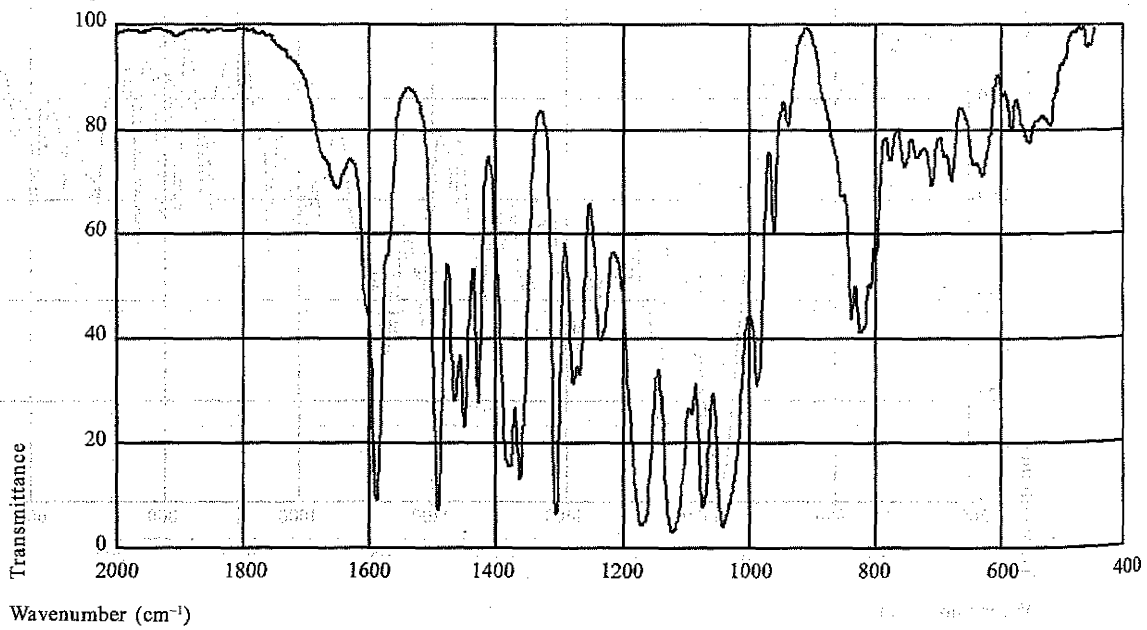
Phase: KBr disc



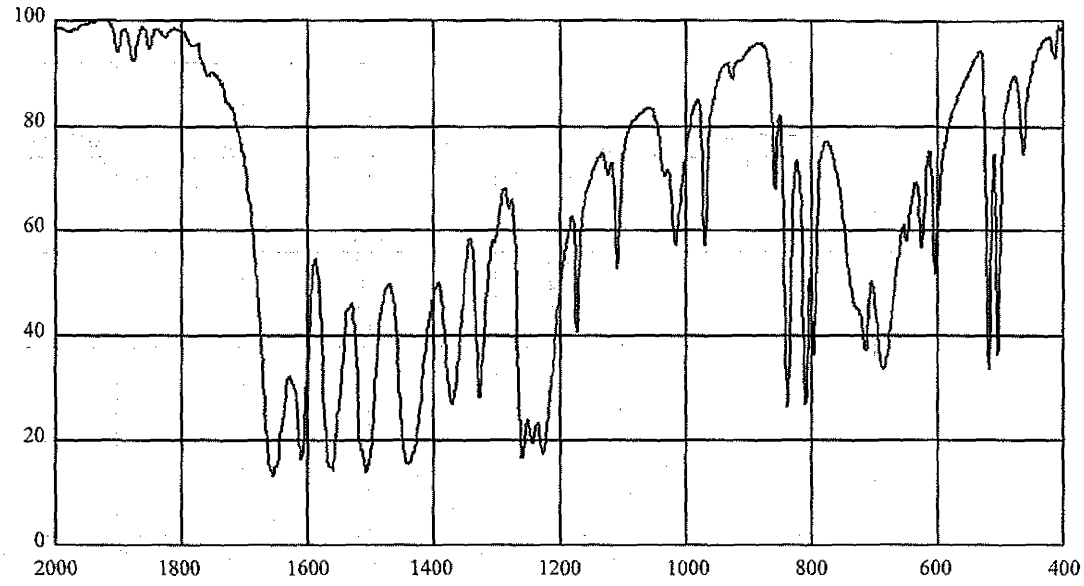
Pantoprazole Sodium

FTIR

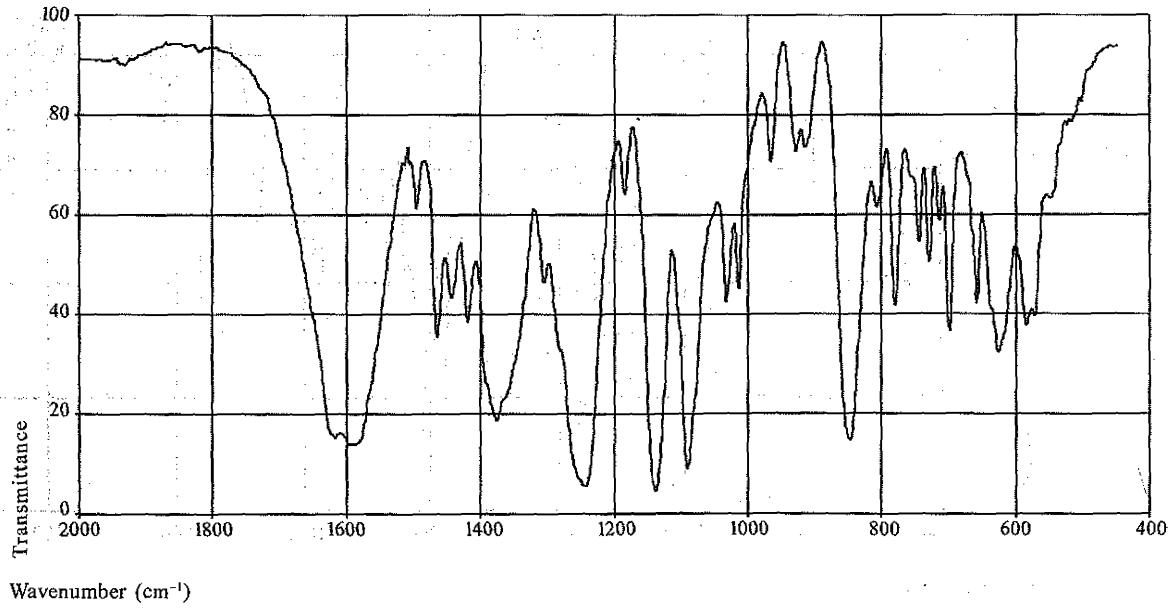
Phase: KBr disc



Paracetamol *FTIR* Phase: KBr disc



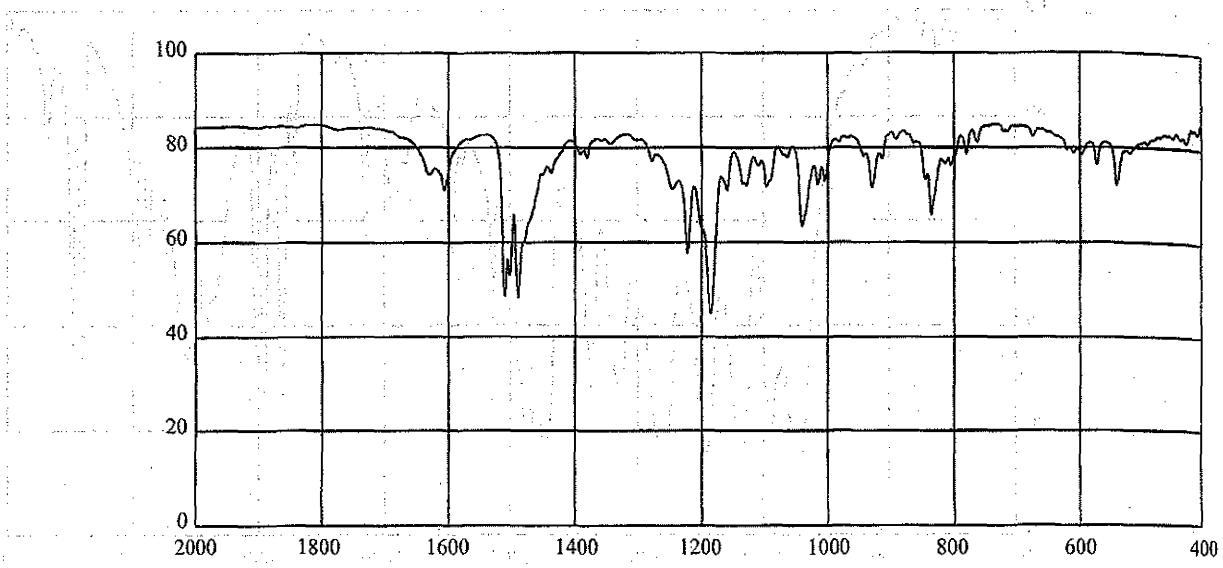
Parecoxib Sodium *FTIR* Phase: KBr disc



Paroxetine Hydrochloride

FTIR

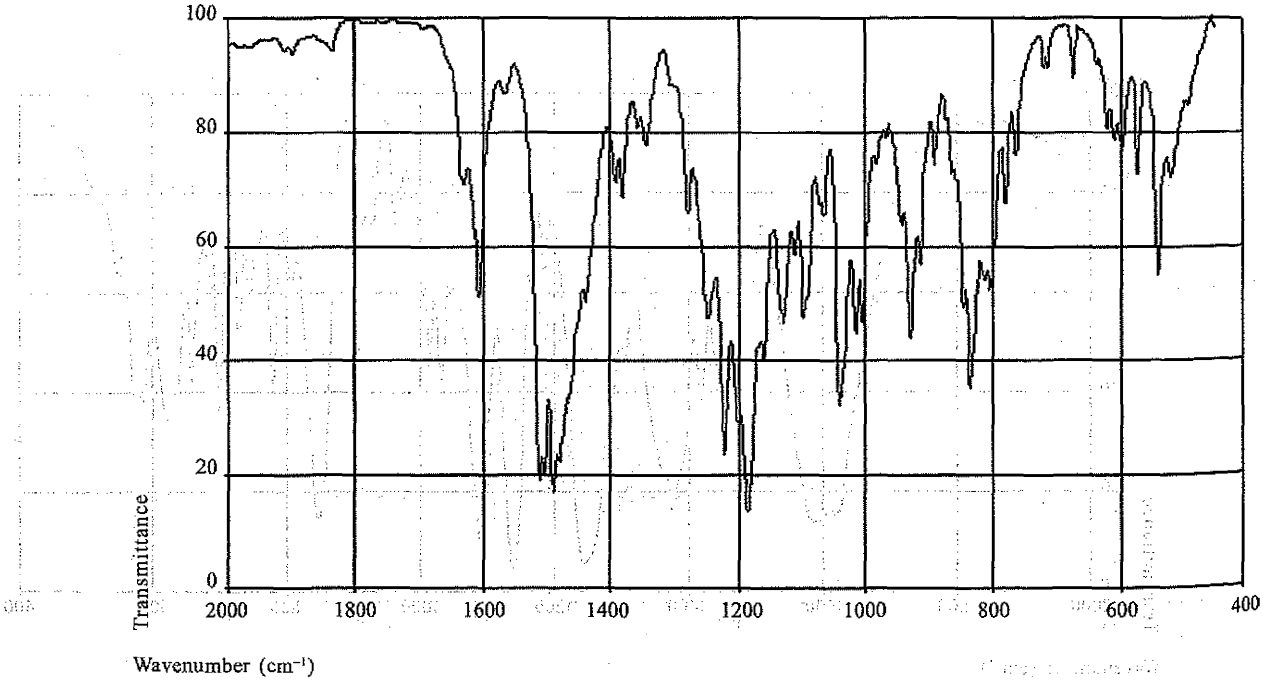
Phase: KBr disc



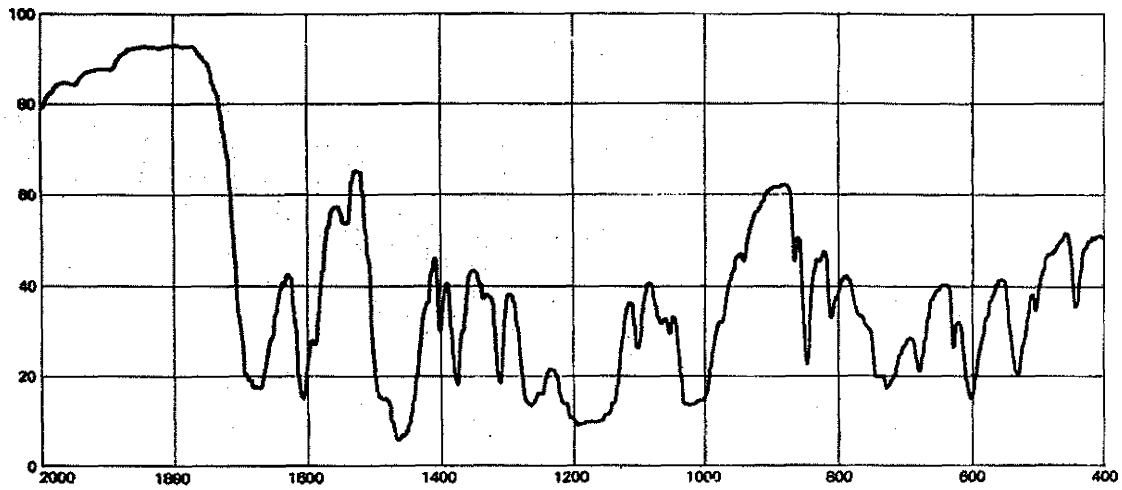
Paroxetine Hydrochloride Hemihydrate

FTIR

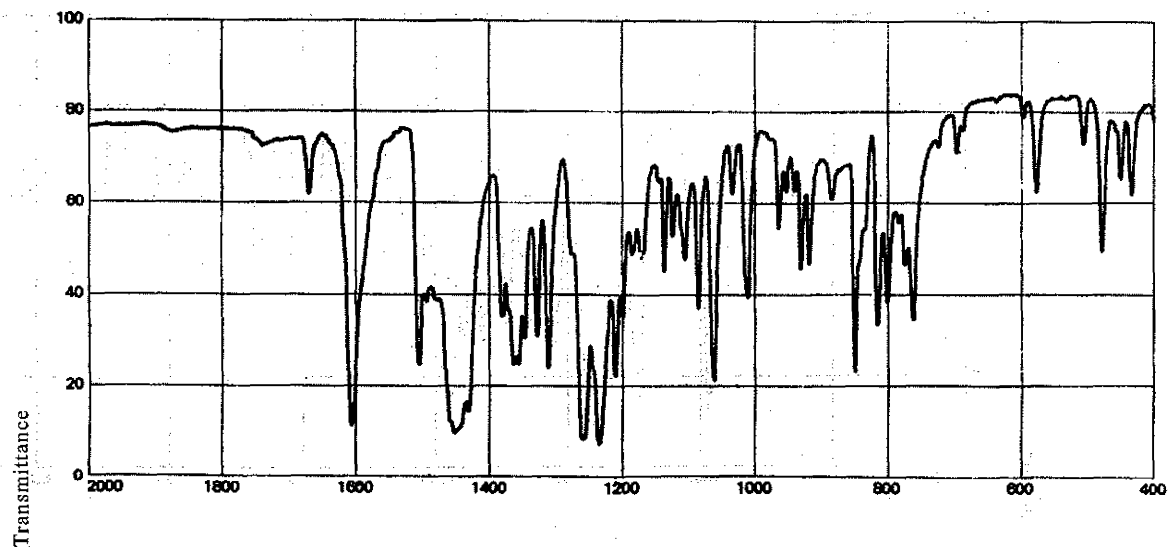
Phase: KBr disc



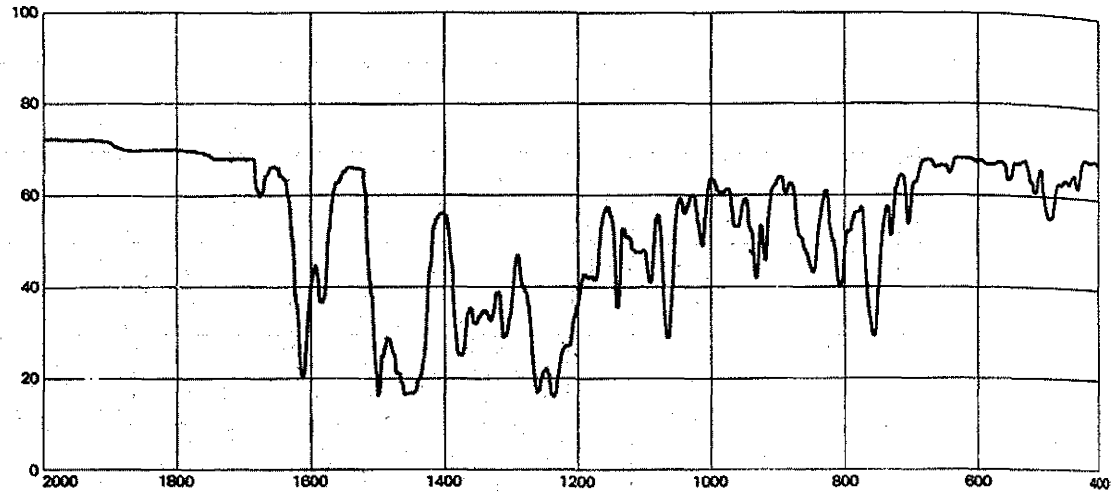
Pentamidine Isethionate *Dispersive IR* Phase: Liquid paraffin mull



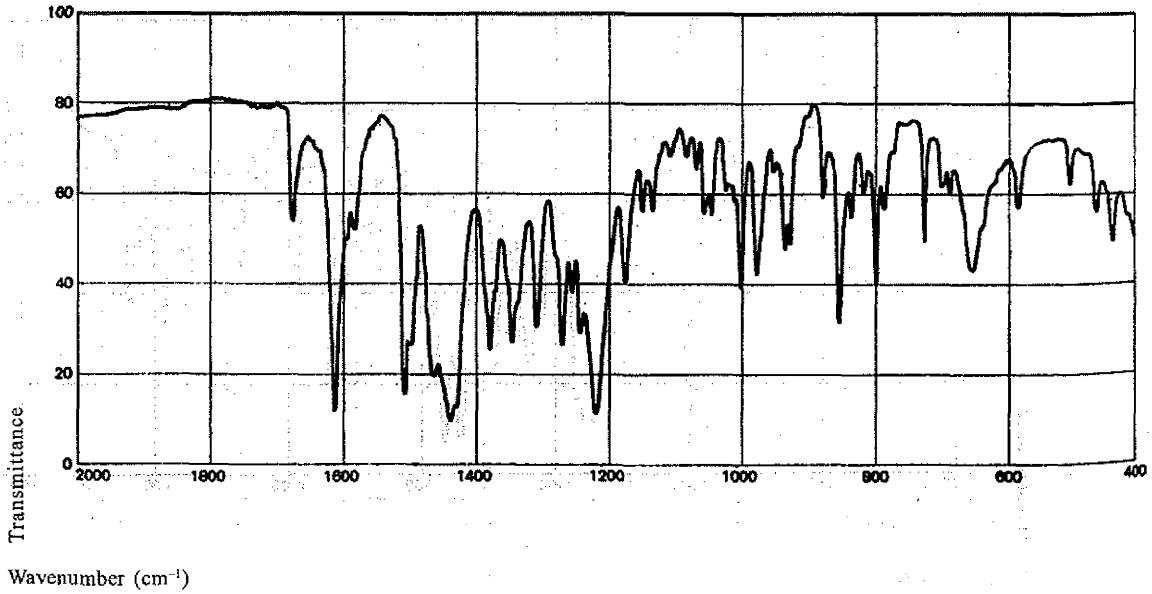
Pentazocine (Form A) *Dispersive IR* Phase: KBr disc



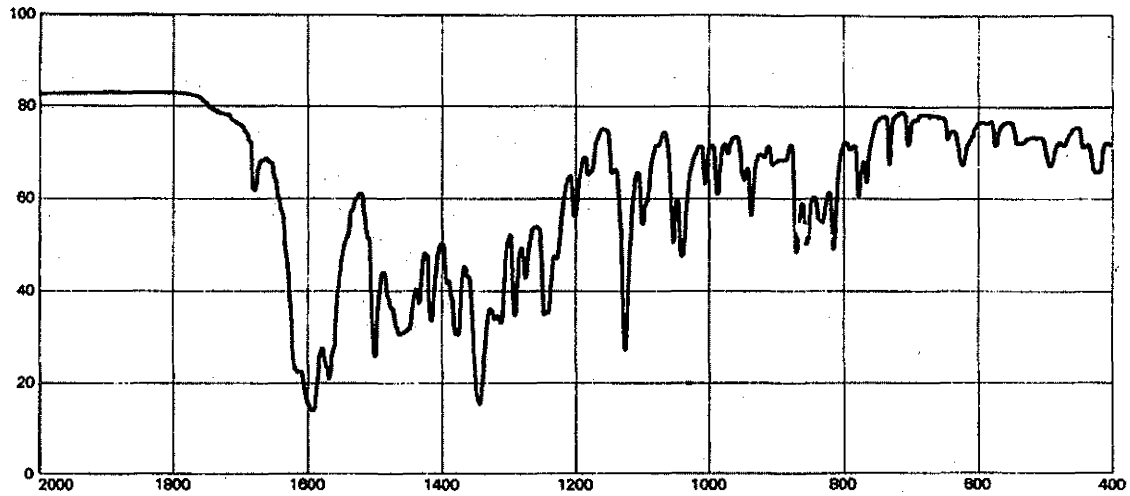
Pentazocine (Form B) *Dispersive IR* Phase: KBr disc



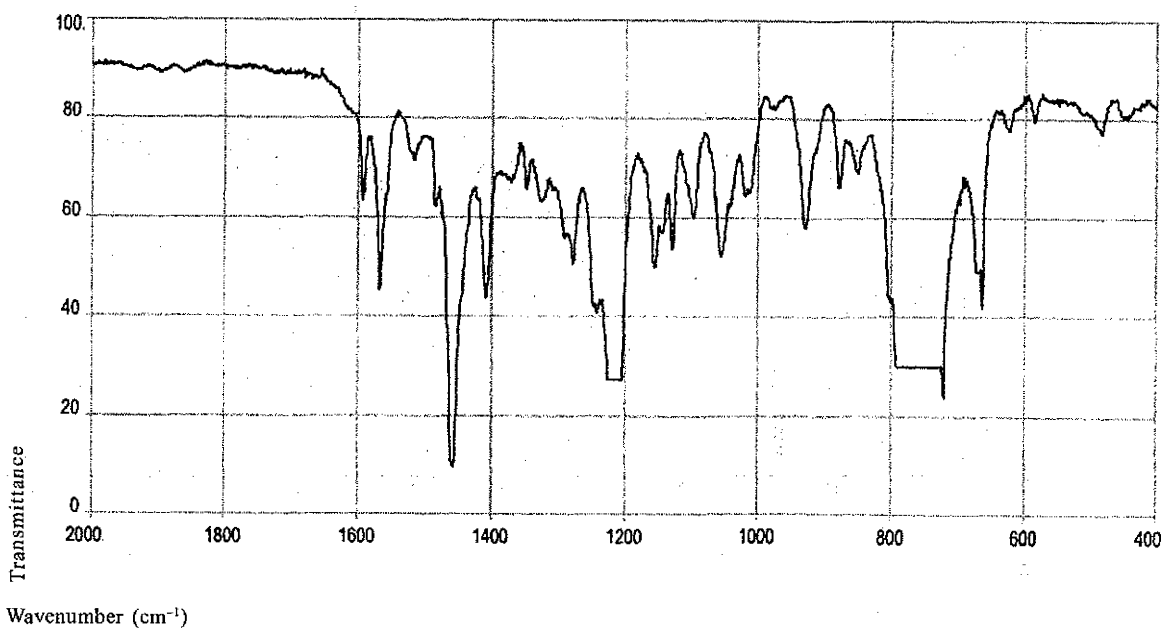
Pentazocine Hydrochloride *Dispersive IR* Phase: KCl disc



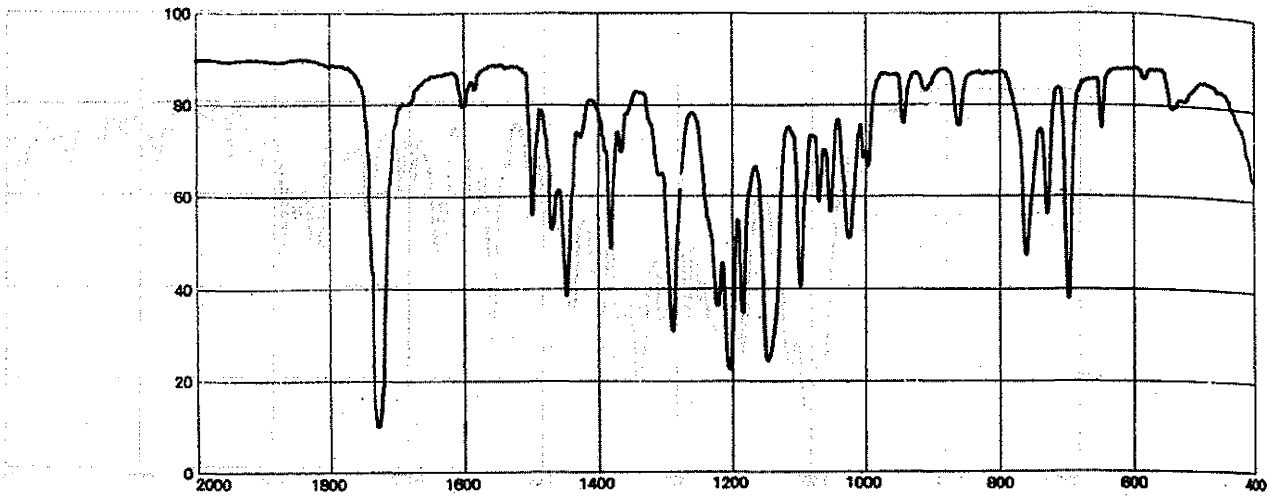
Pentazocine Lactate *Dispersive IR* Phase: KBr disc



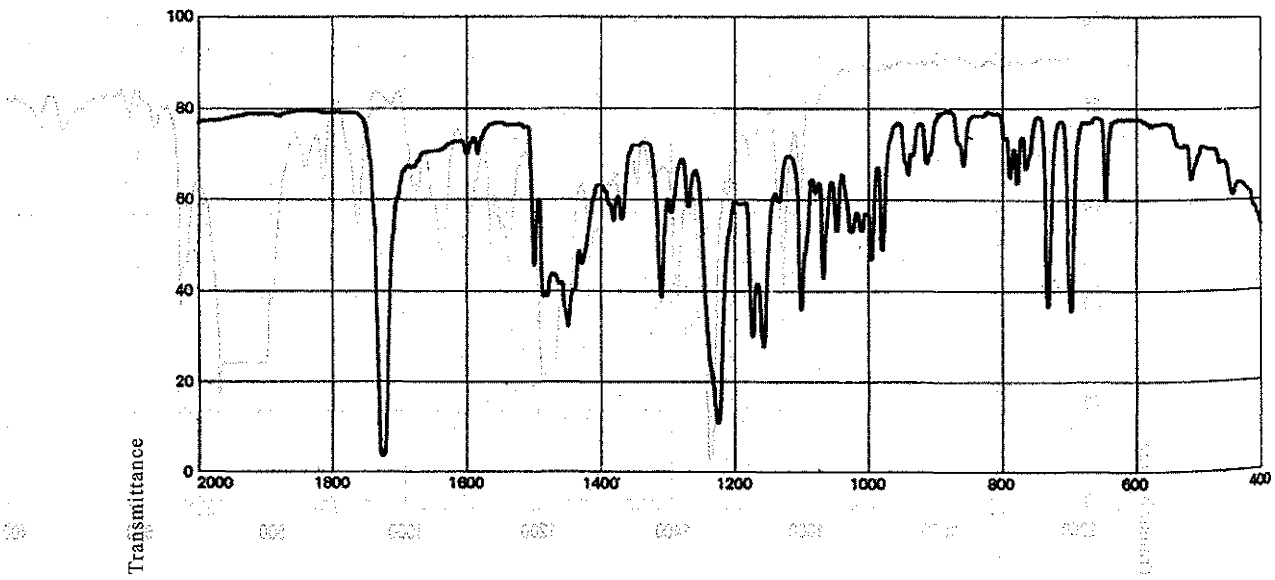
Perphenazine *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



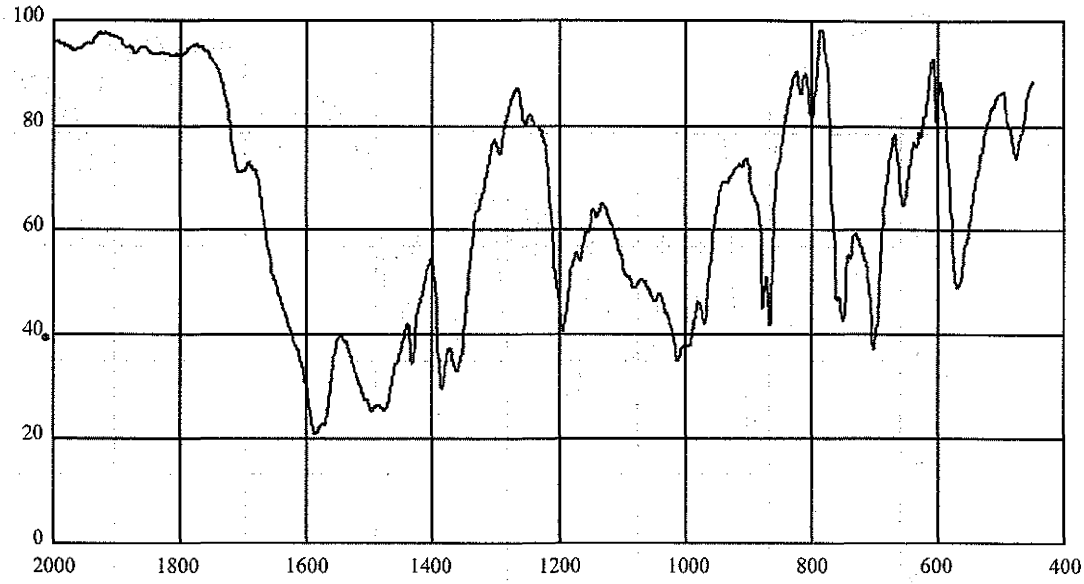
Pethidine *Dispersive IR* Phase: Thin film



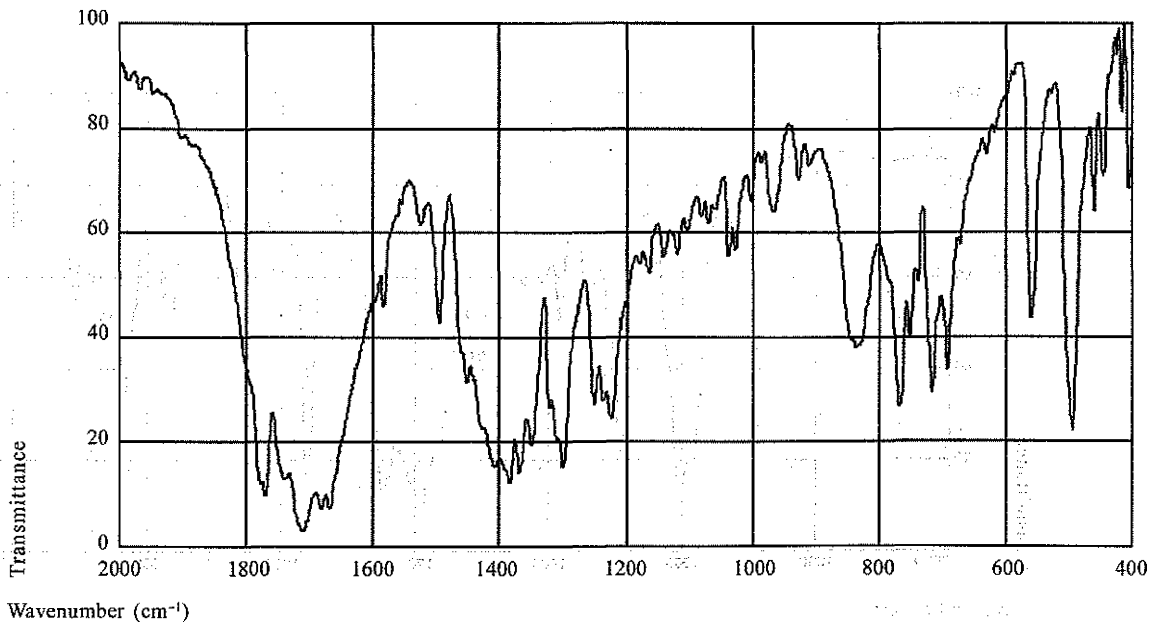
Pethidine Hydrochloride *Dispersive IR* Phase: KCl disc



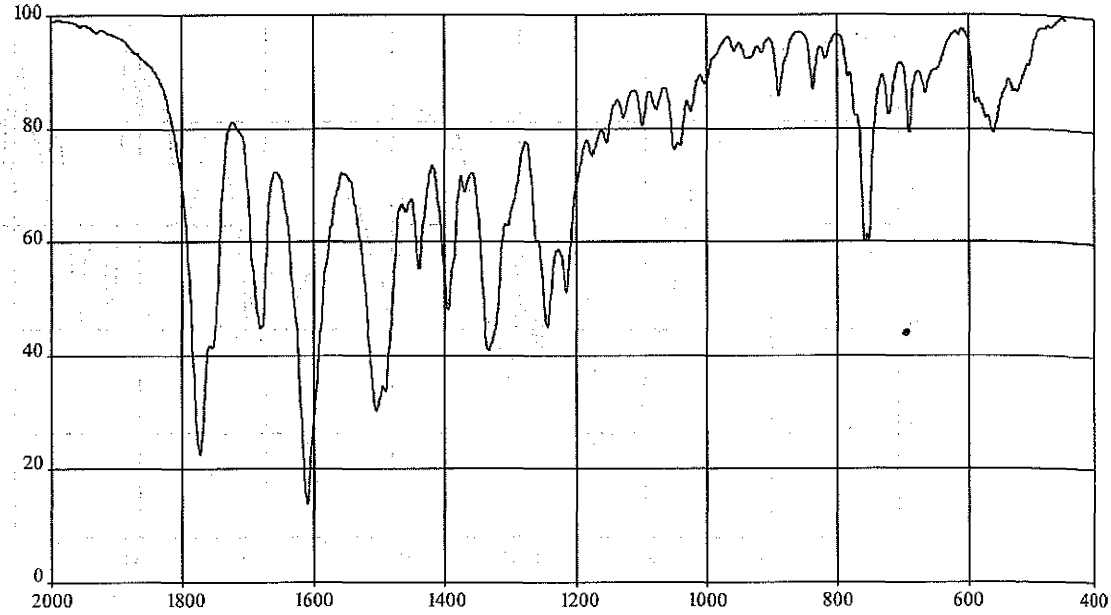
Pheniramine Maleate *FTIR* Phase: Liquid paraffin mull



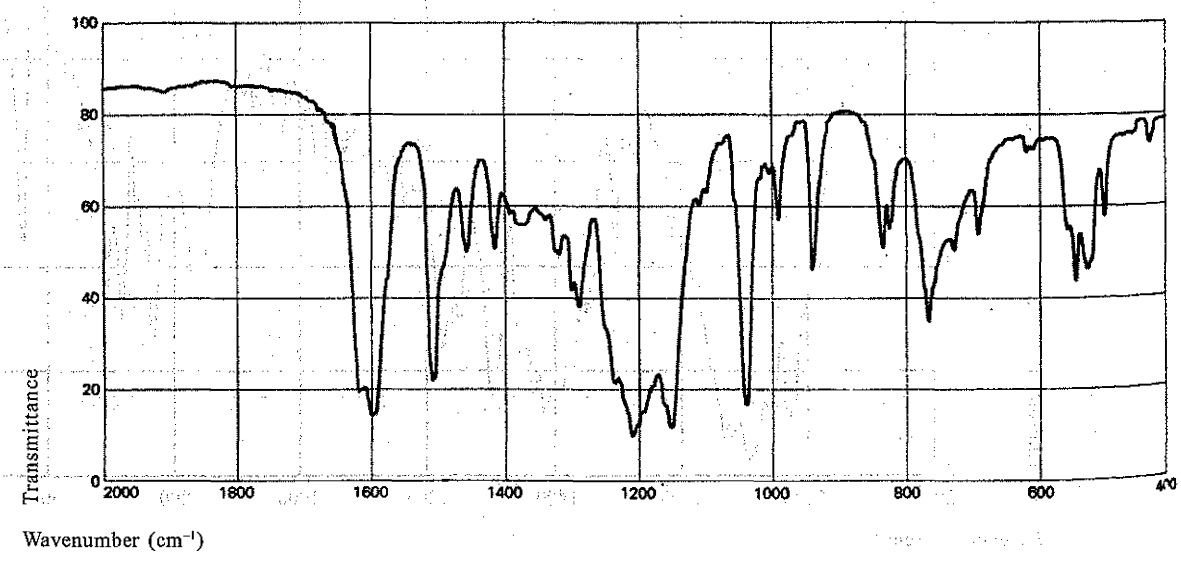
Phenobarbitone *FTIR* Phase: KBr disc



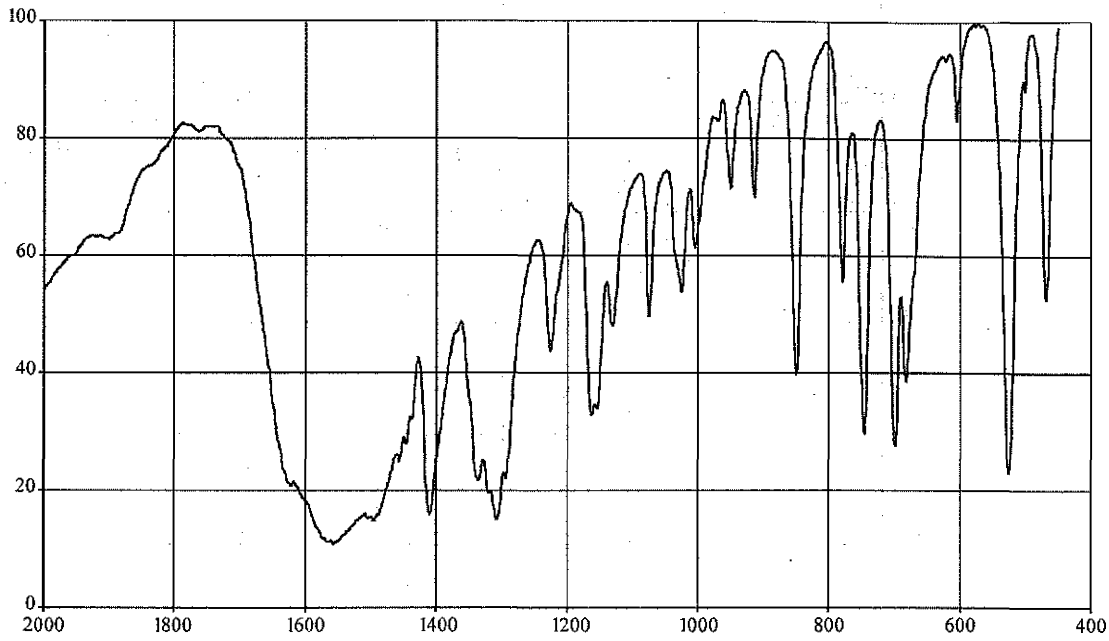
Phenoxymethylpenicillin Potassium *Dispersive IR* Phase: KBr disc



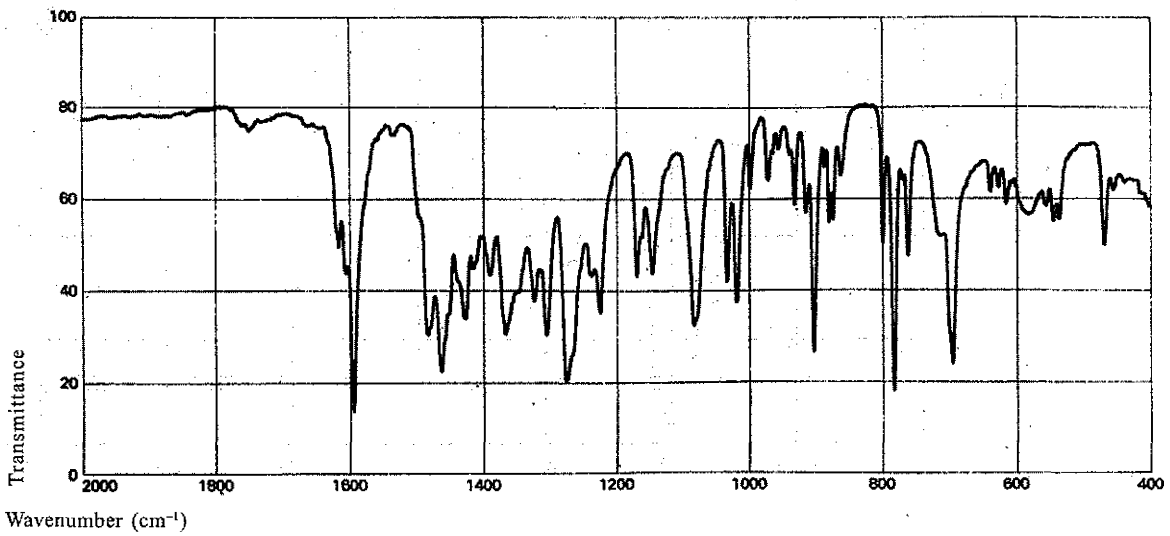
Phentolamine Mesylate *Dispersive IR* Phase: KBr disc



Phenylalanine *Dispersive IR* Phase: KBr disc

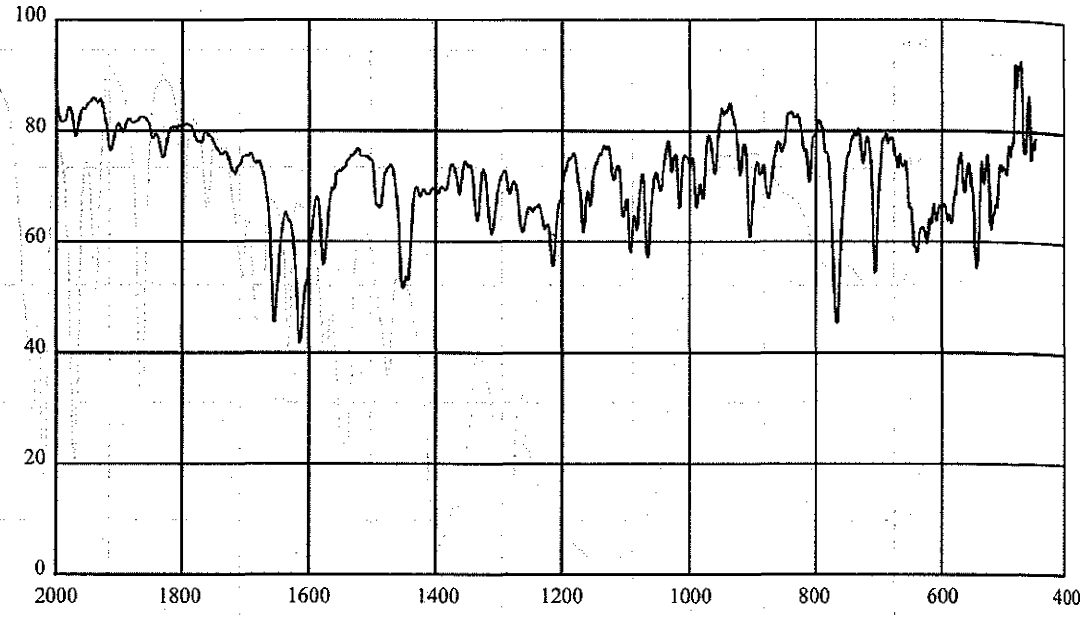


Phenylephrine Hydrochloride *Dispersive IR* Phase: KCl disc



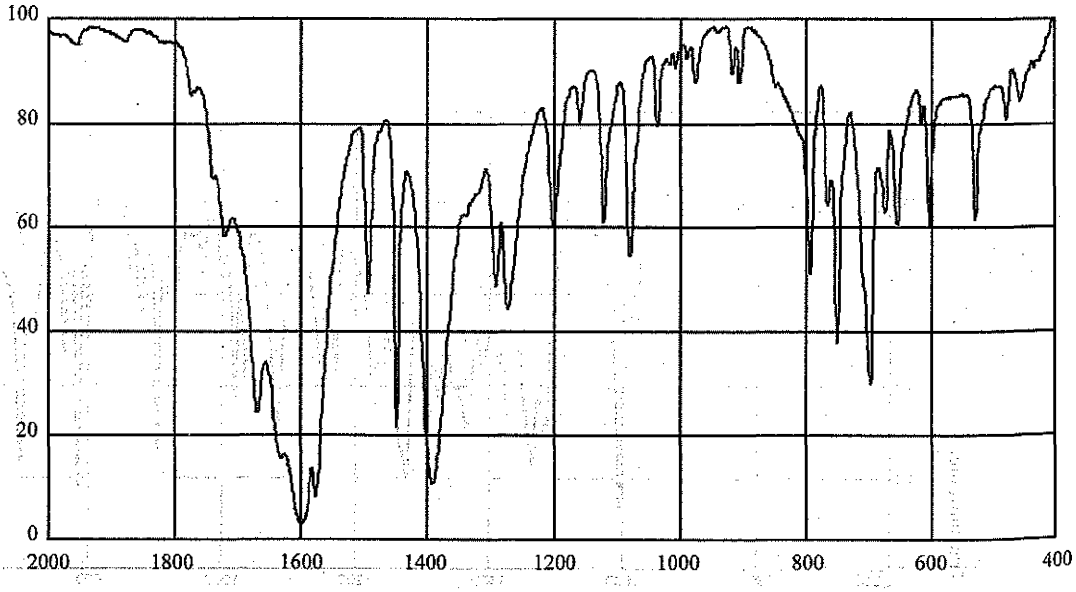
Phenyramidol Hydrochloride

FTIR Phase: KBr disc

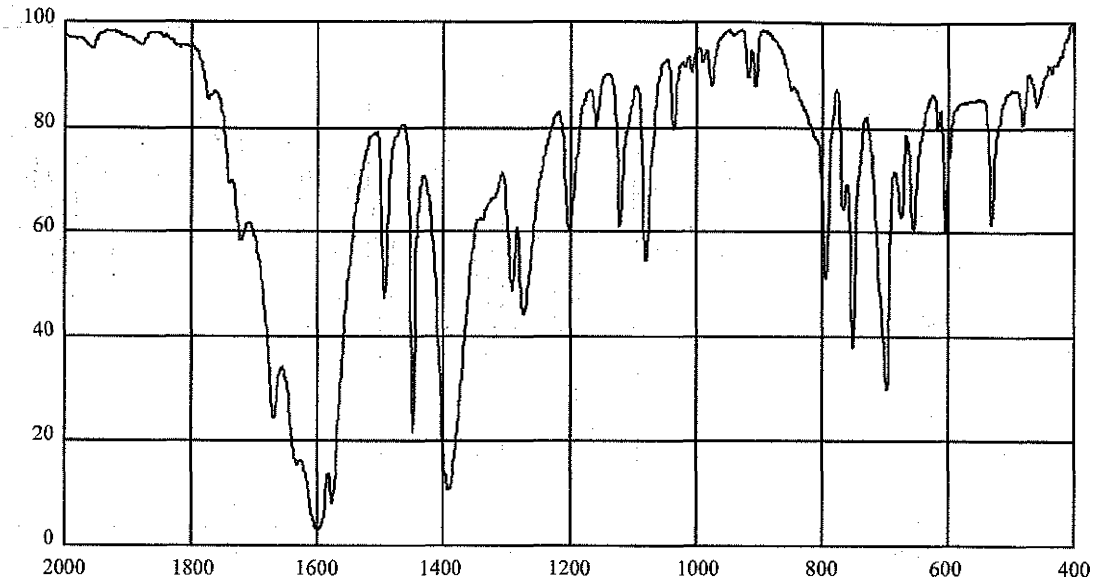


Phenytoin

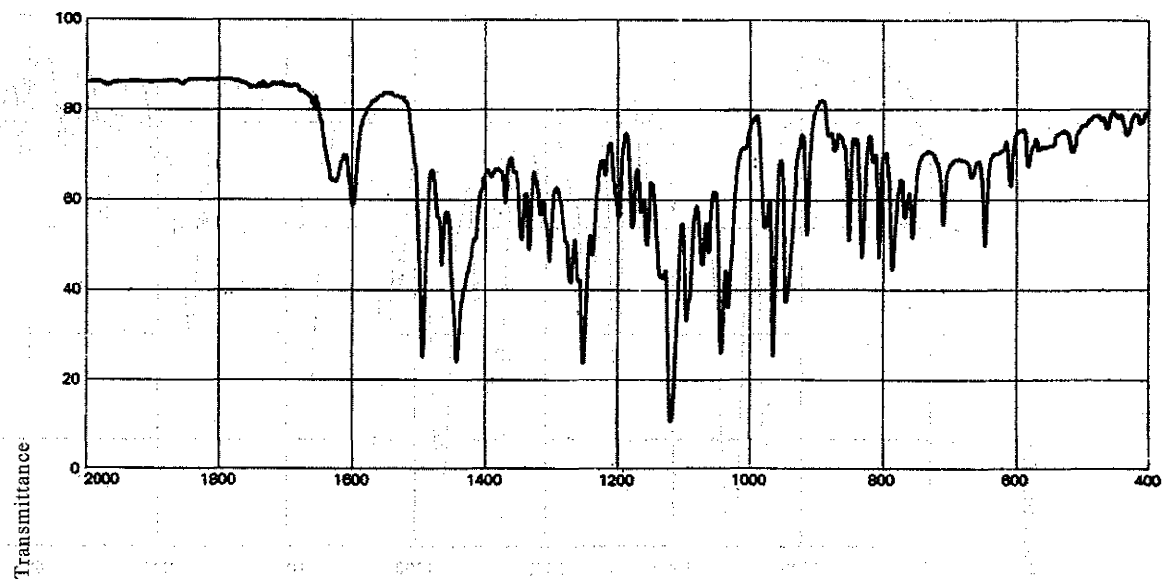
FTIR Phase: KBr disc



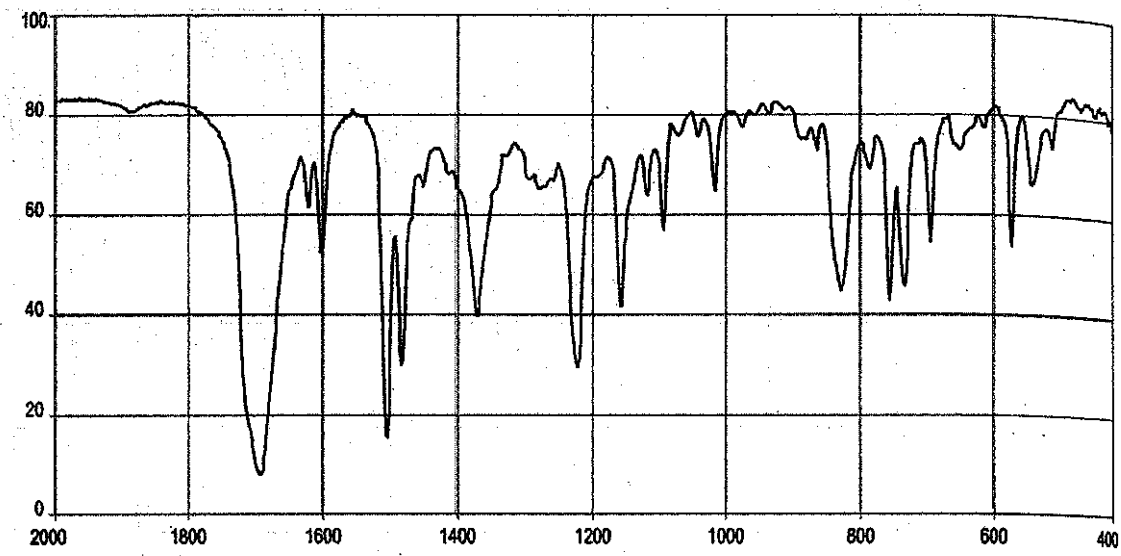
Phenytoin Sodium *FTIR* Phase: KBr disc



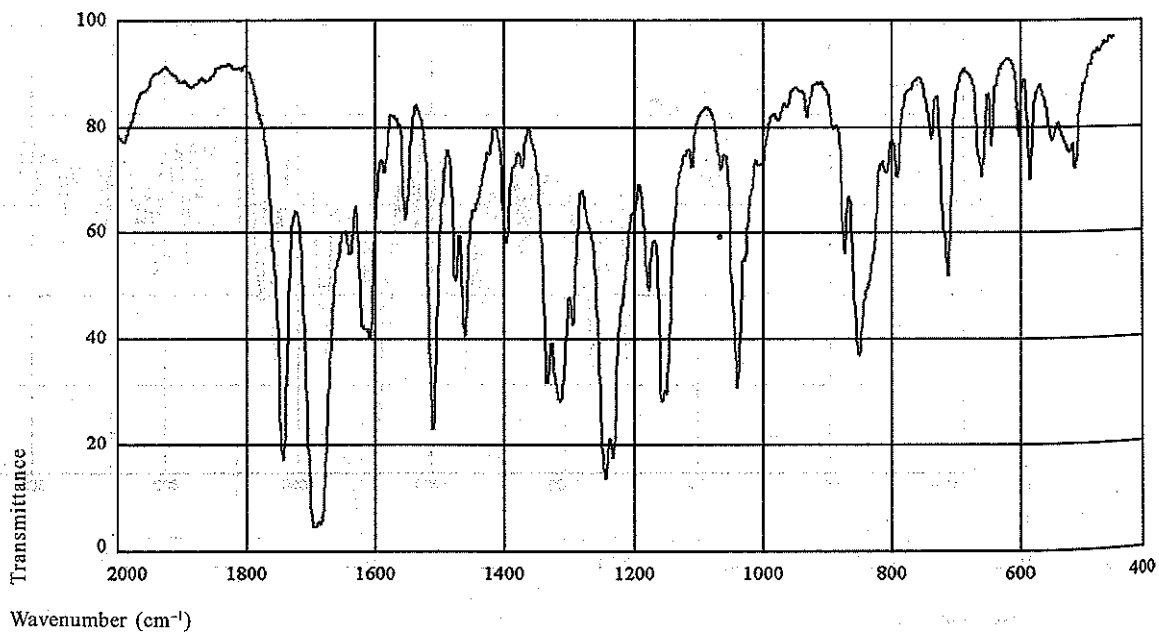
Pholcodine *Dispersive IR* Phase: KBr disc



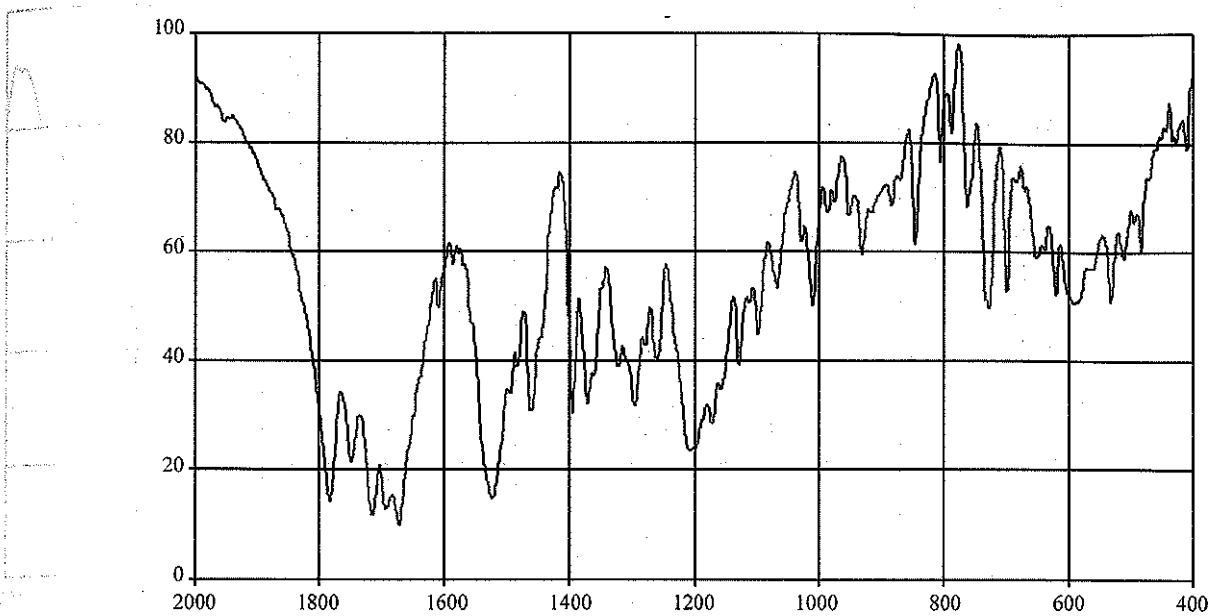
Pimozide *FTIR* Phase: KBr disc



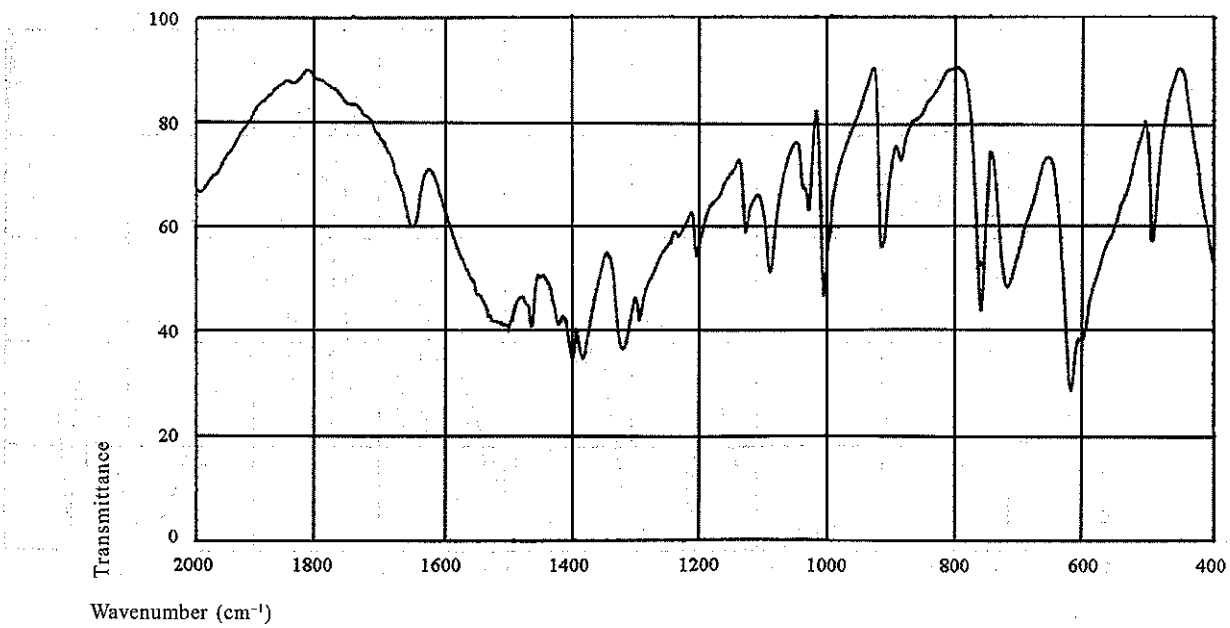
Pioglitazone Hydrochloride *FTIR* Phase: KBr disc



Piperacillin *FTIR* Phase: KBr disc



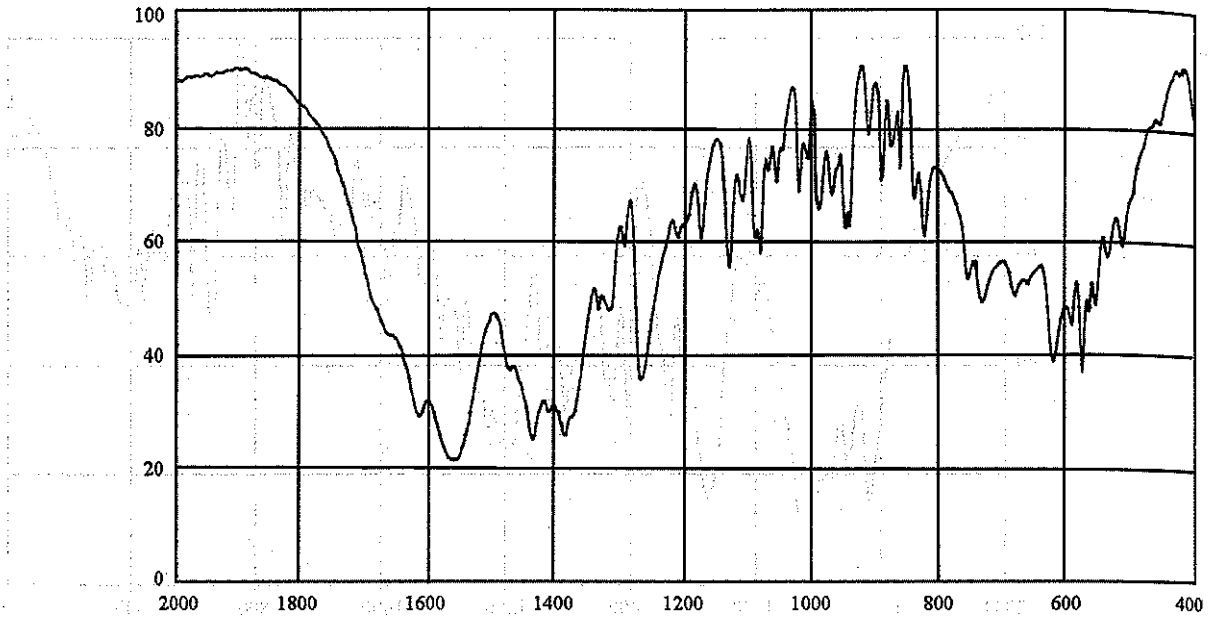
Piperazine Adipate *FTIR* Phase: KBr disc



Piperazine Citrate

FTIR

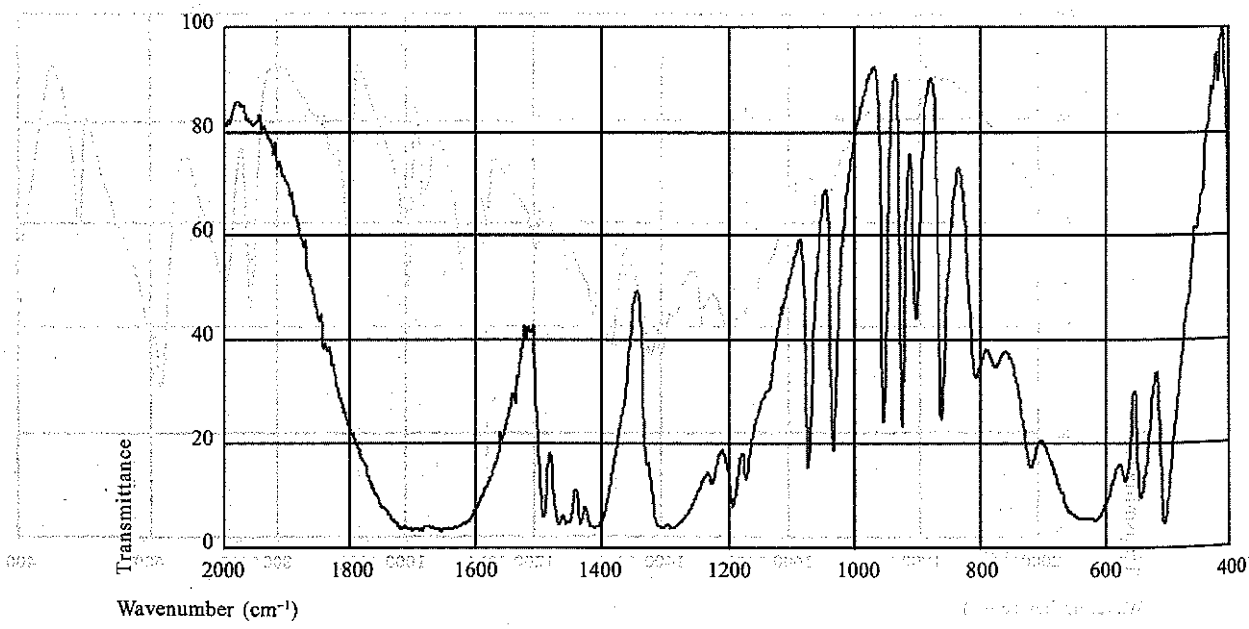
Phase: KBr disc



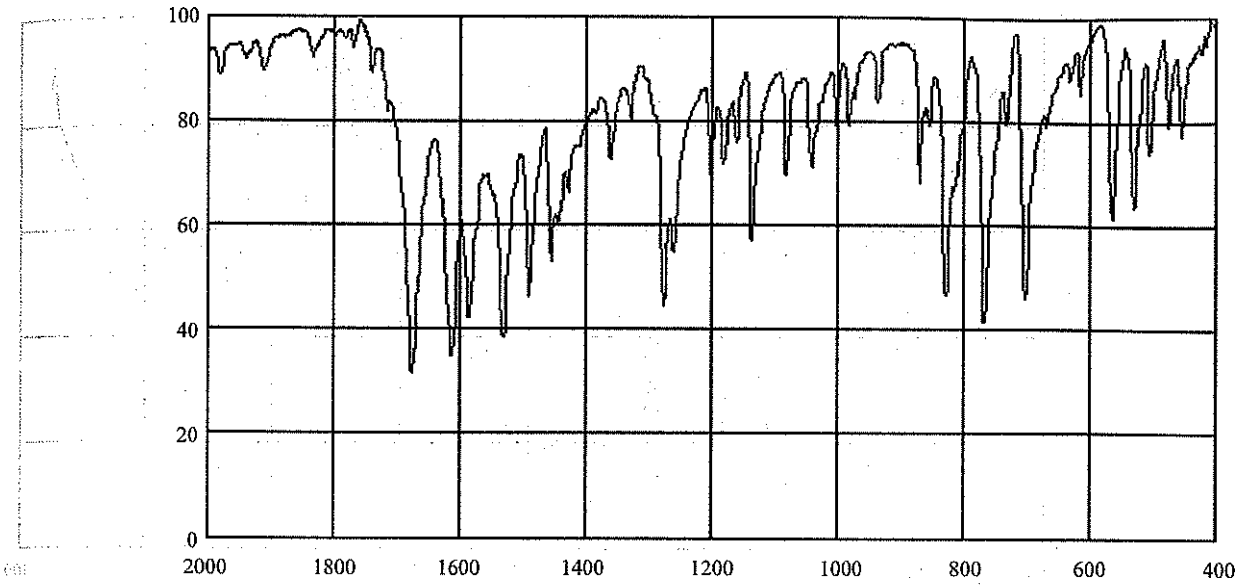
Piracetam

FTIR

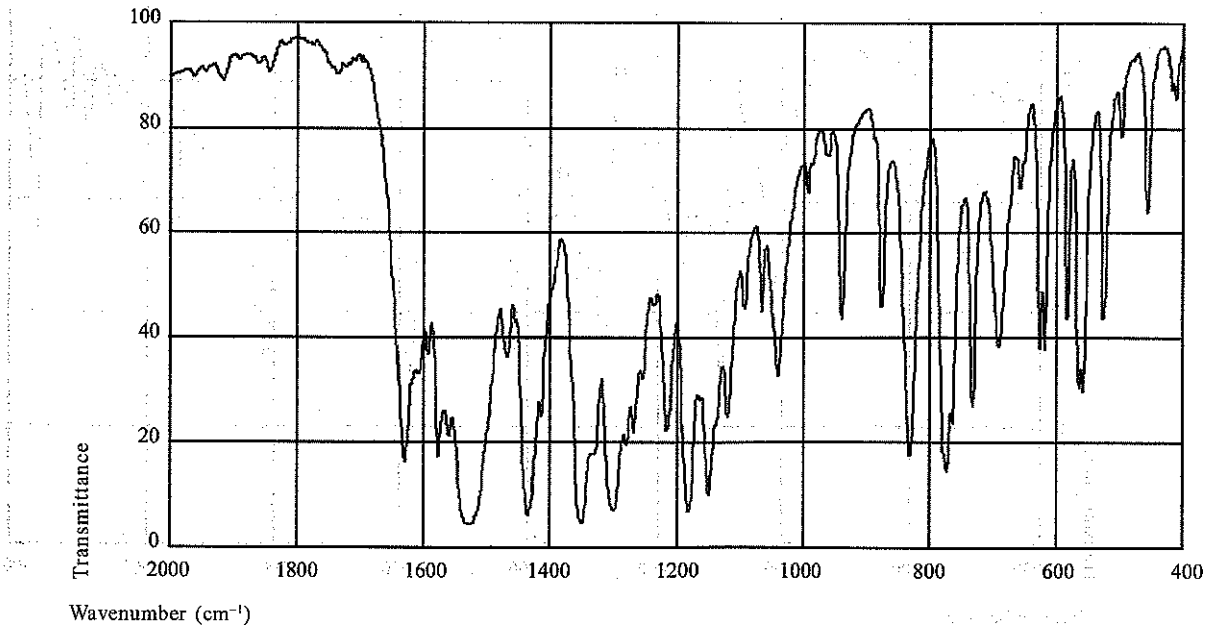
Phase: KBr disc



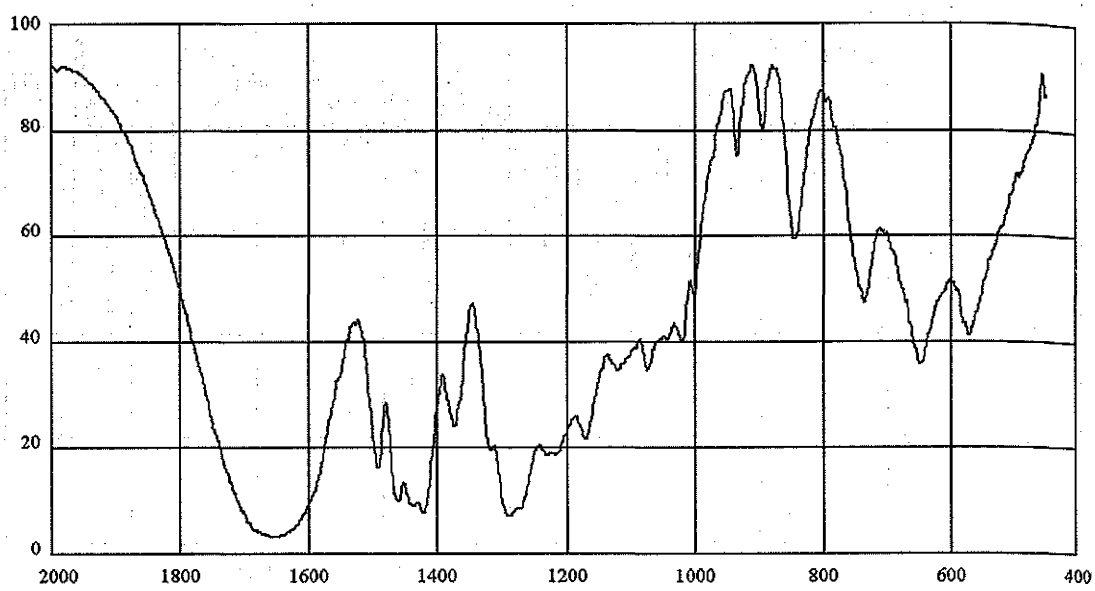
Pirfenidone *FTIR* Phase: KBr disc



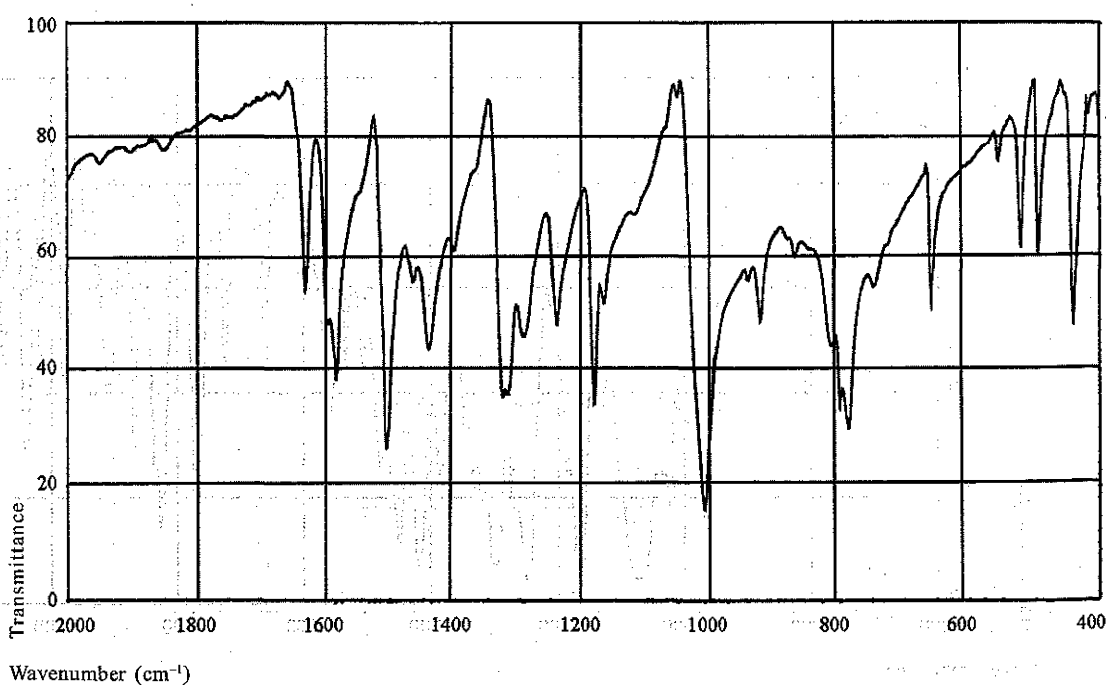
Piroxicam *FTIR* Phase: KBr disc



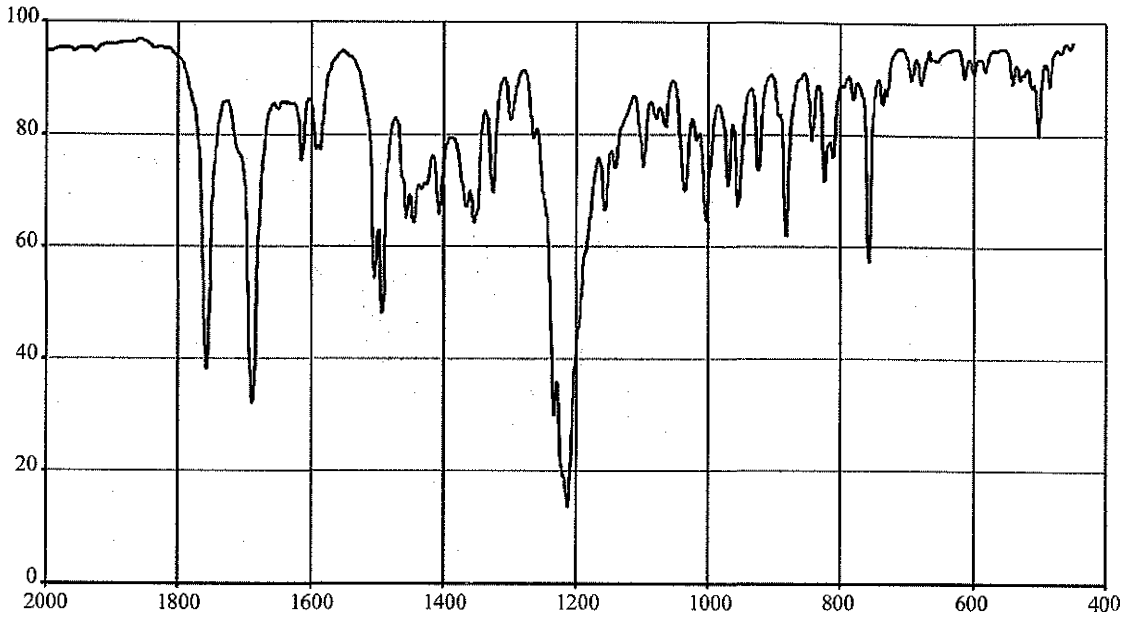
Povidone *FTIR* Phase: KBr disc



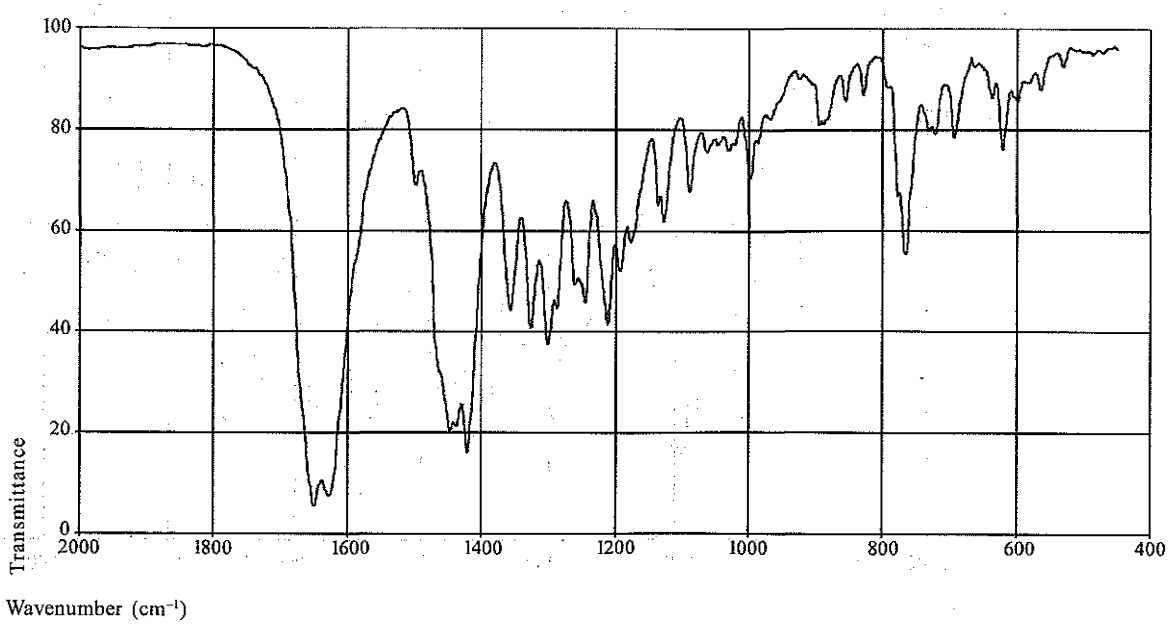
Pralidoxime Chloride *FTIR* Phase: KBr disc



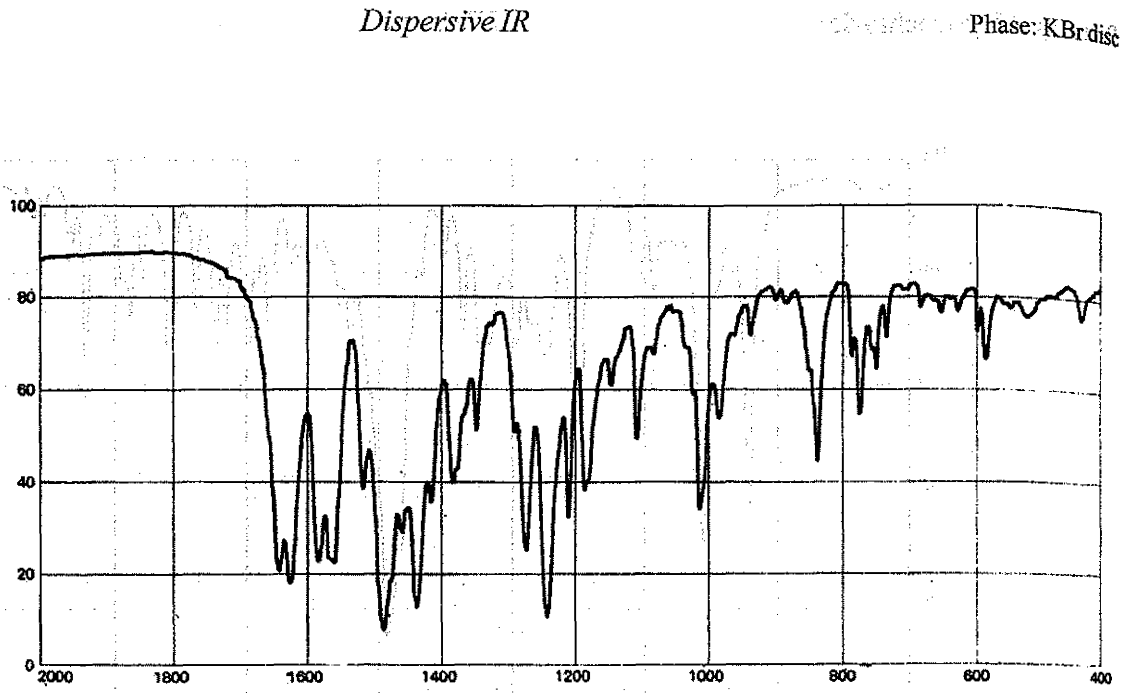
Prasugrel Hydrochloride *FTIR* Phase: KBr disc



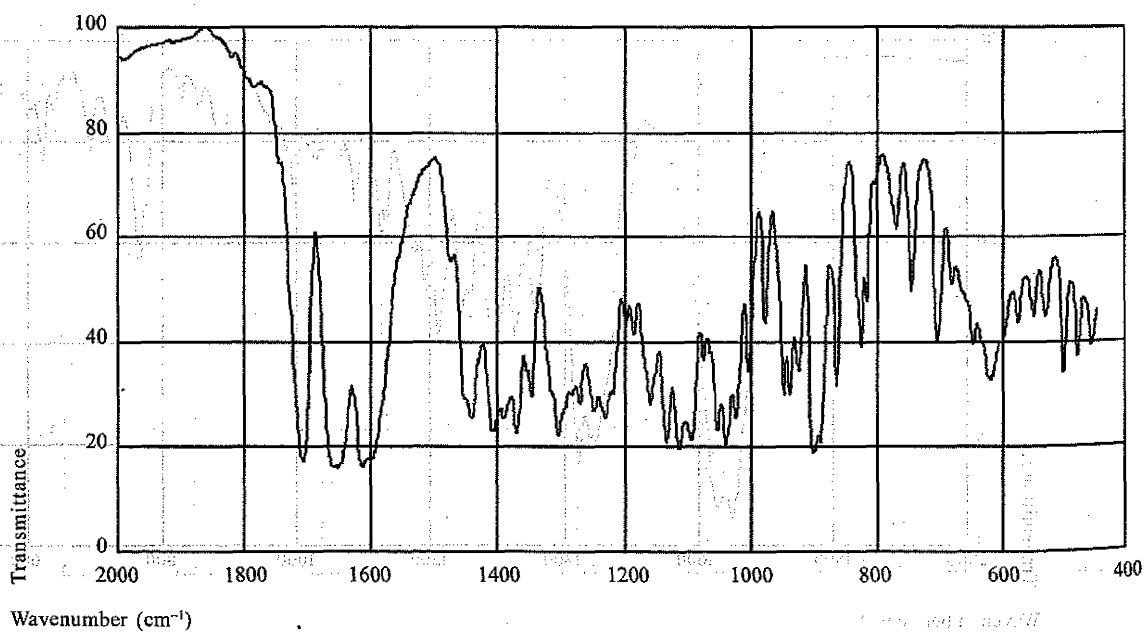
Praziquantel *FTIR* Phase: KBr disc



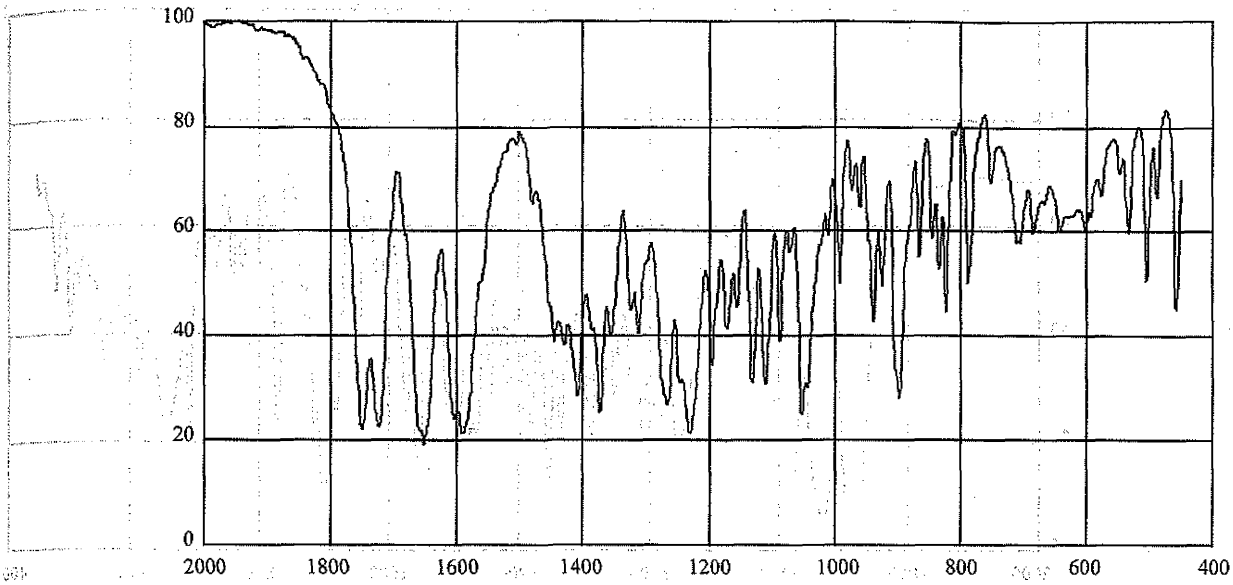
Prazosin *Dispersive IR* Phase: KBr disc



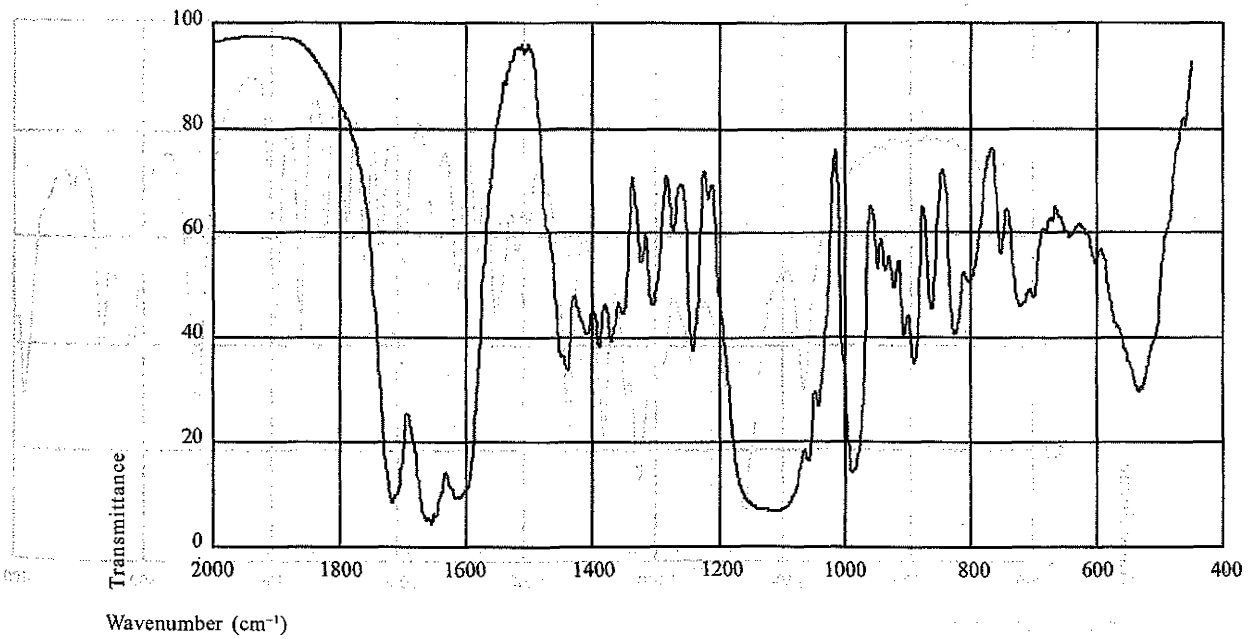
Prednisolone *FTIR* Phase: KBr disc



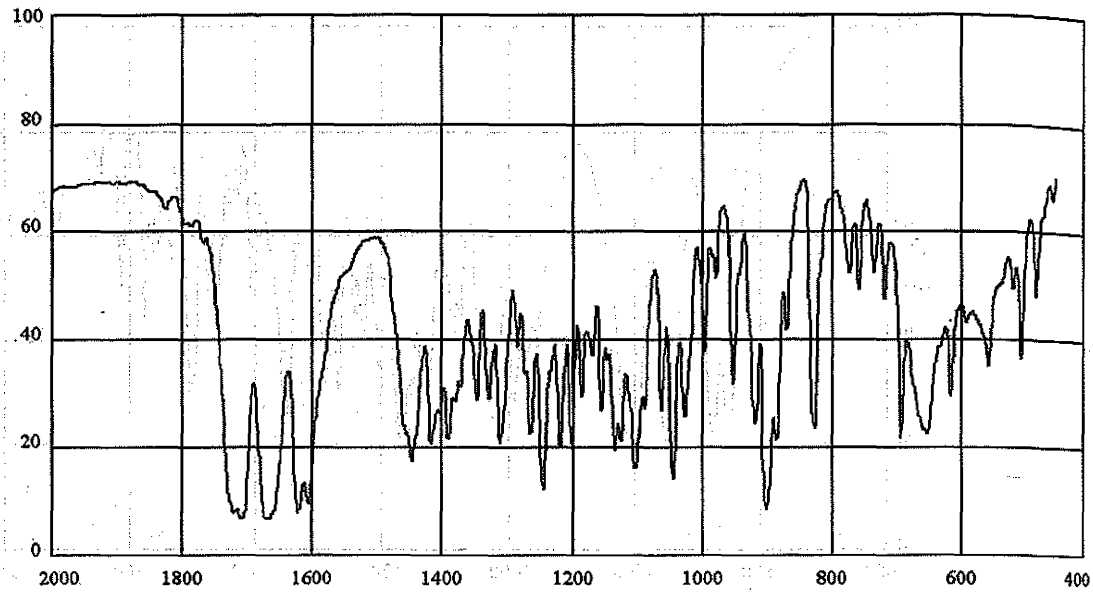
Prednisolone Acetate *FTIR* Phase: KBr disc



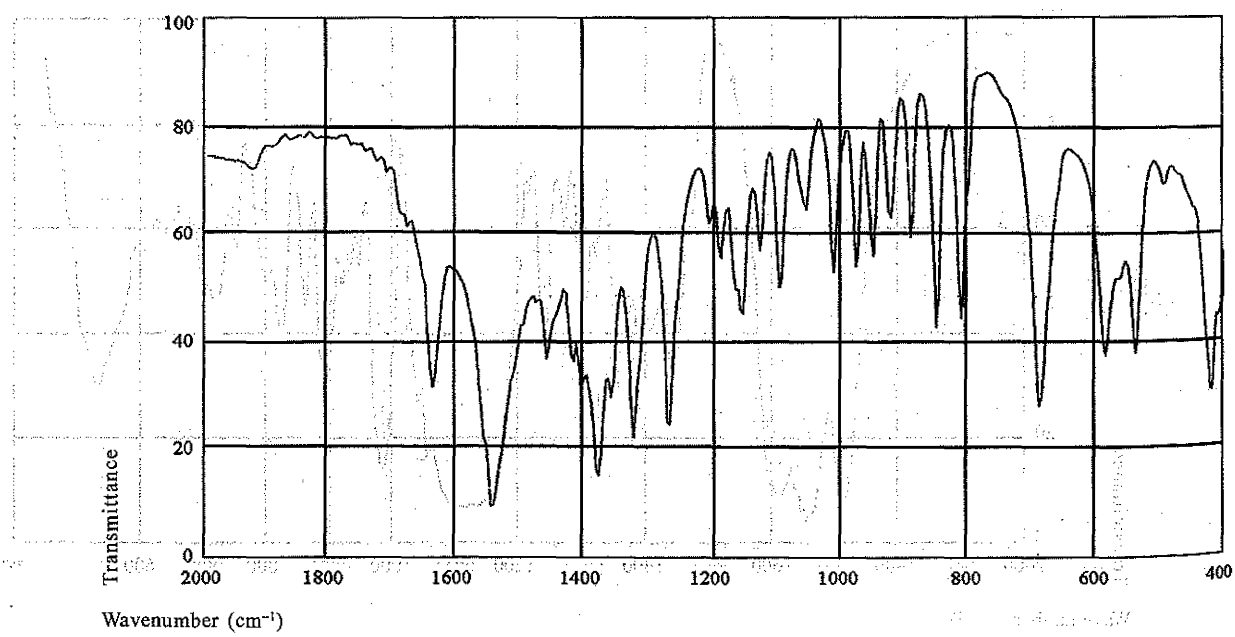
Prednisolone Sodium Phosphate *FTIR* Phase: KBr disc



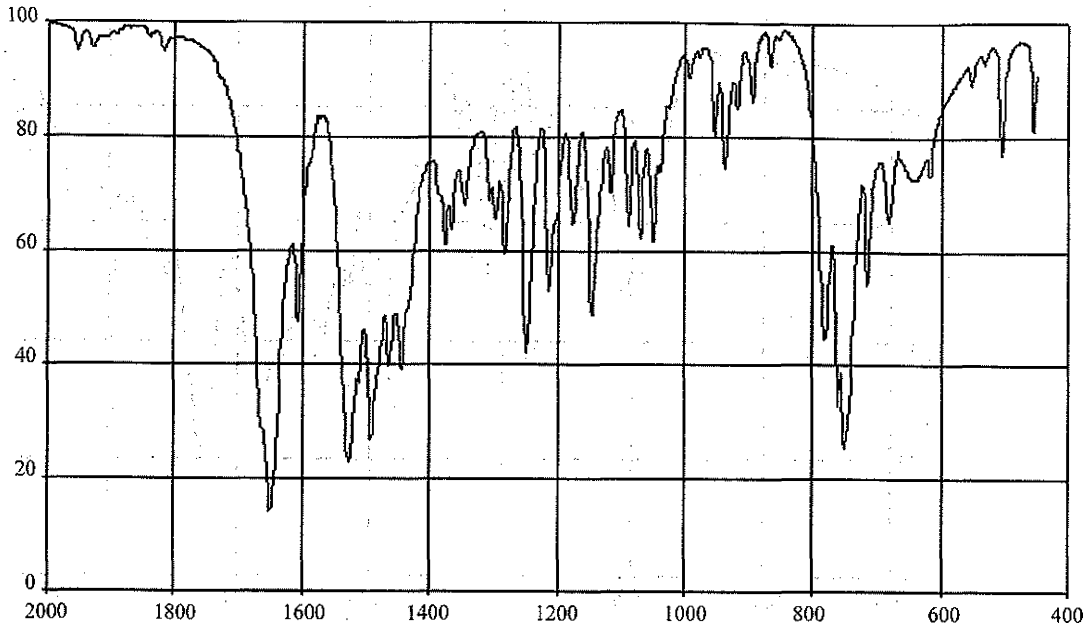
Prednisone *FTIR* Phase: KBr disc



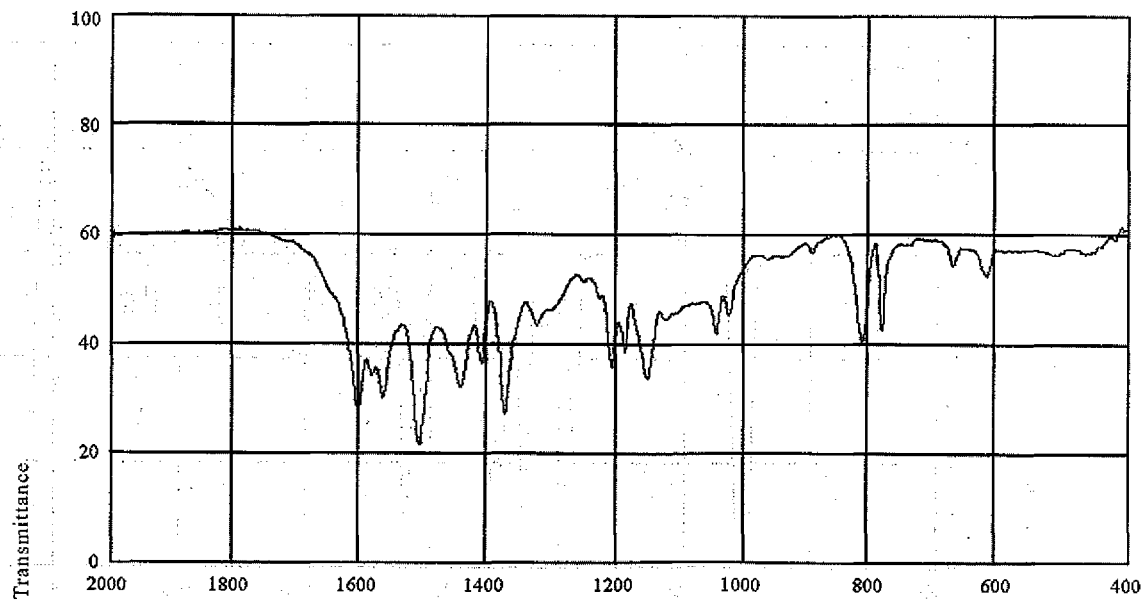
Pregabalin *Dispersive IR* Phase: KBr disc



Prilocaine *FTIR* Phase: KBr disc



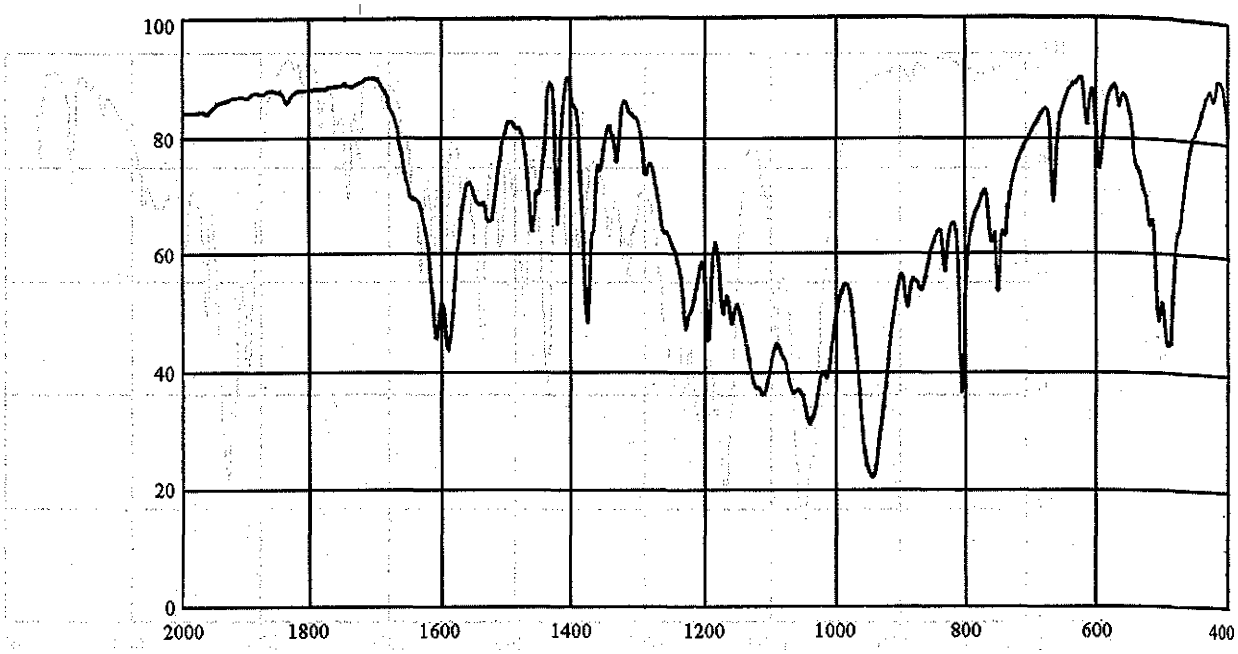
Primaquine *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



Primaquine Phosphate

FTIR

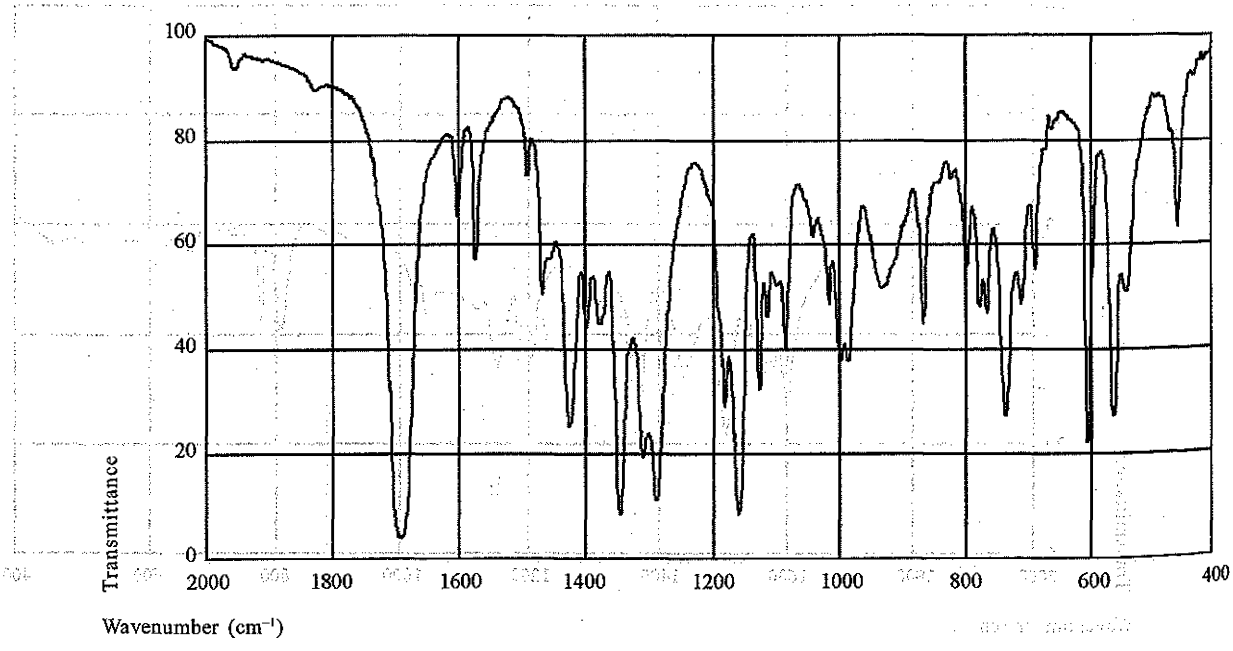
Phase: KBr disc



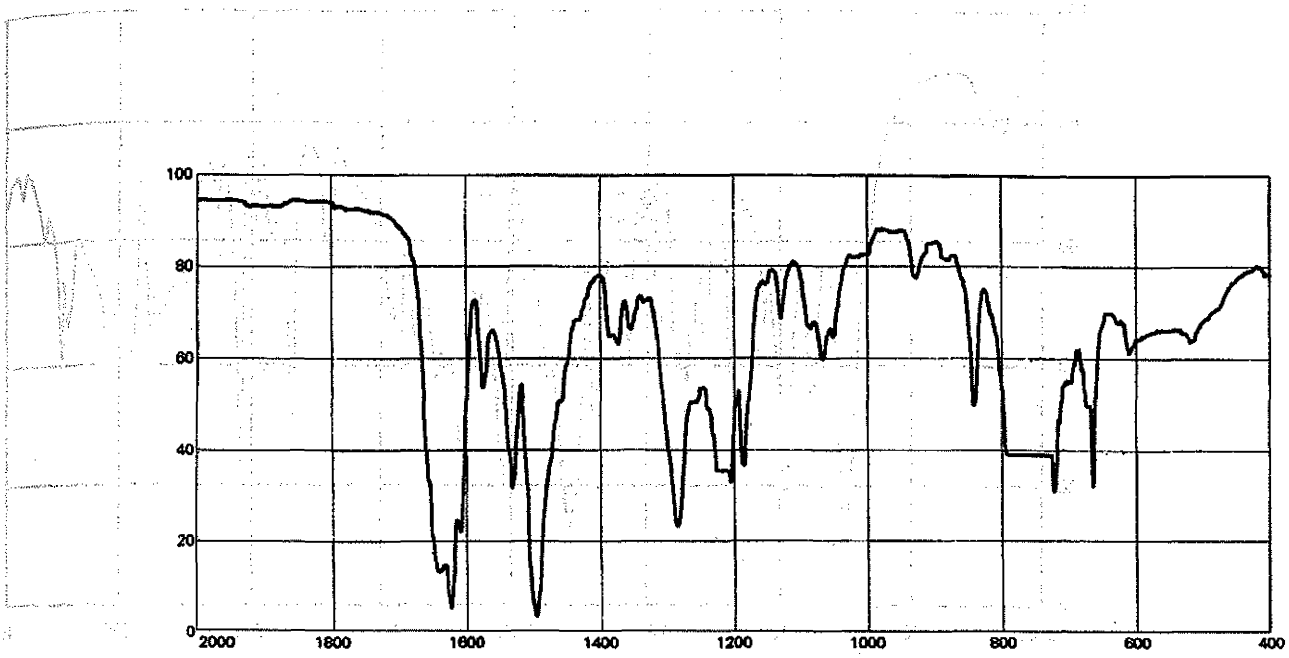
Probenecid

FTIR

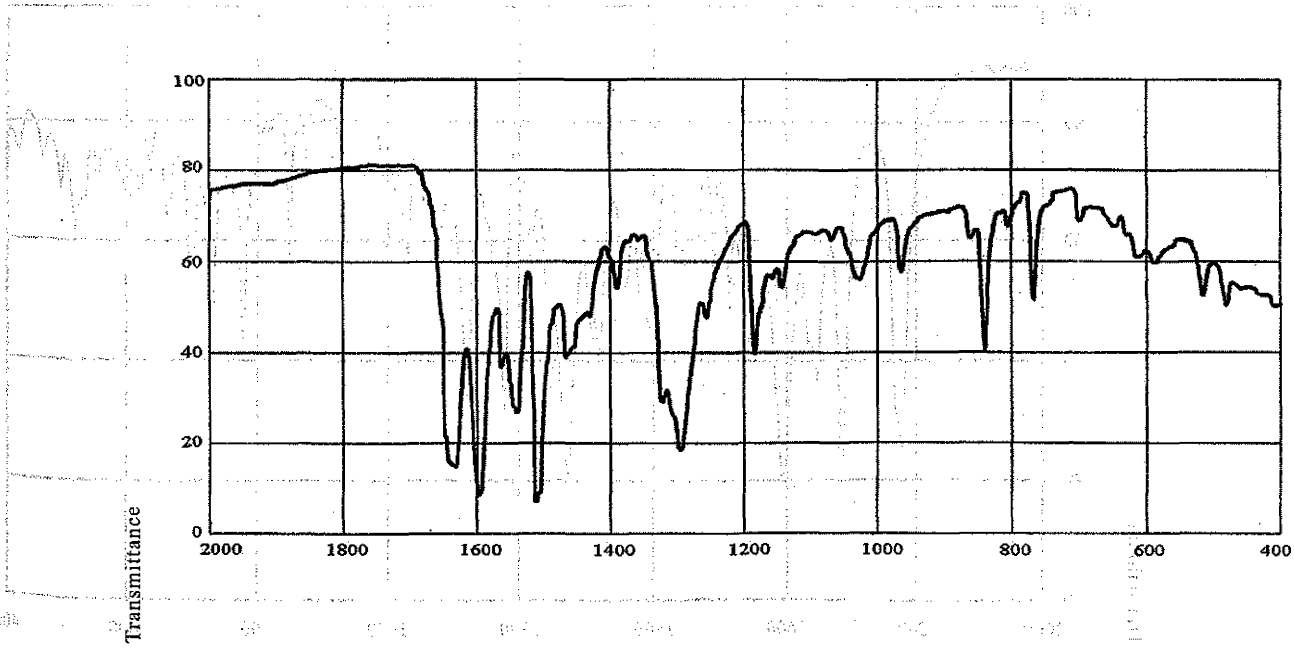
Phase: KBr disc



Procainamide *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



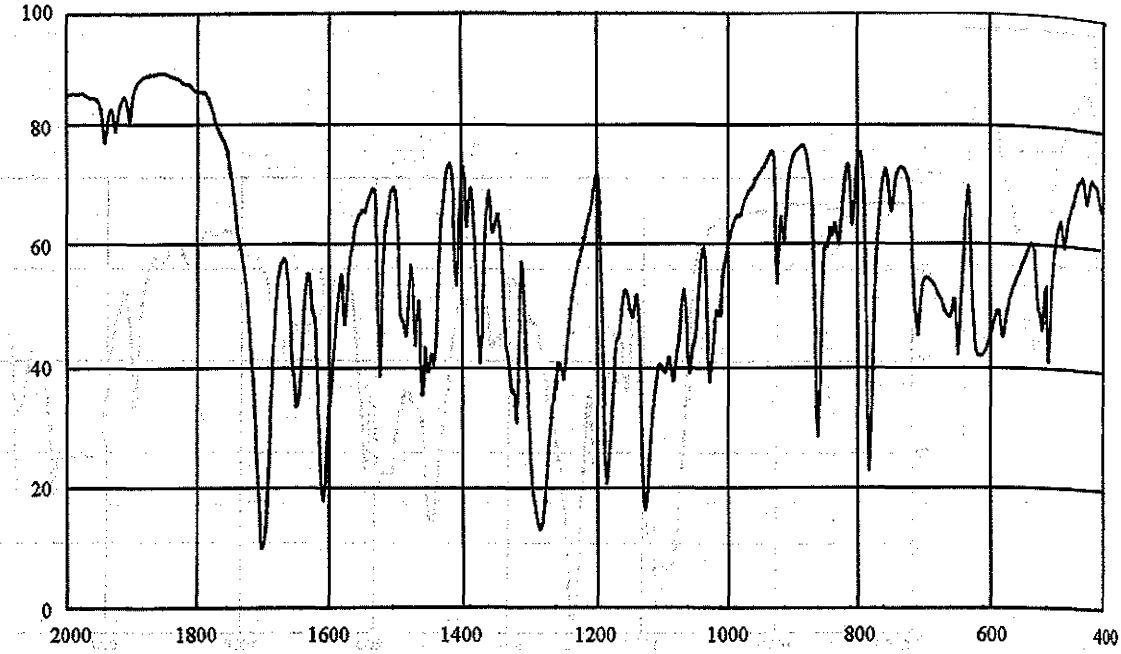
Procainamide Hydrochloride *Dispersive IR* Phase: KCl disc



Procaine Hydrochloride

FTIR

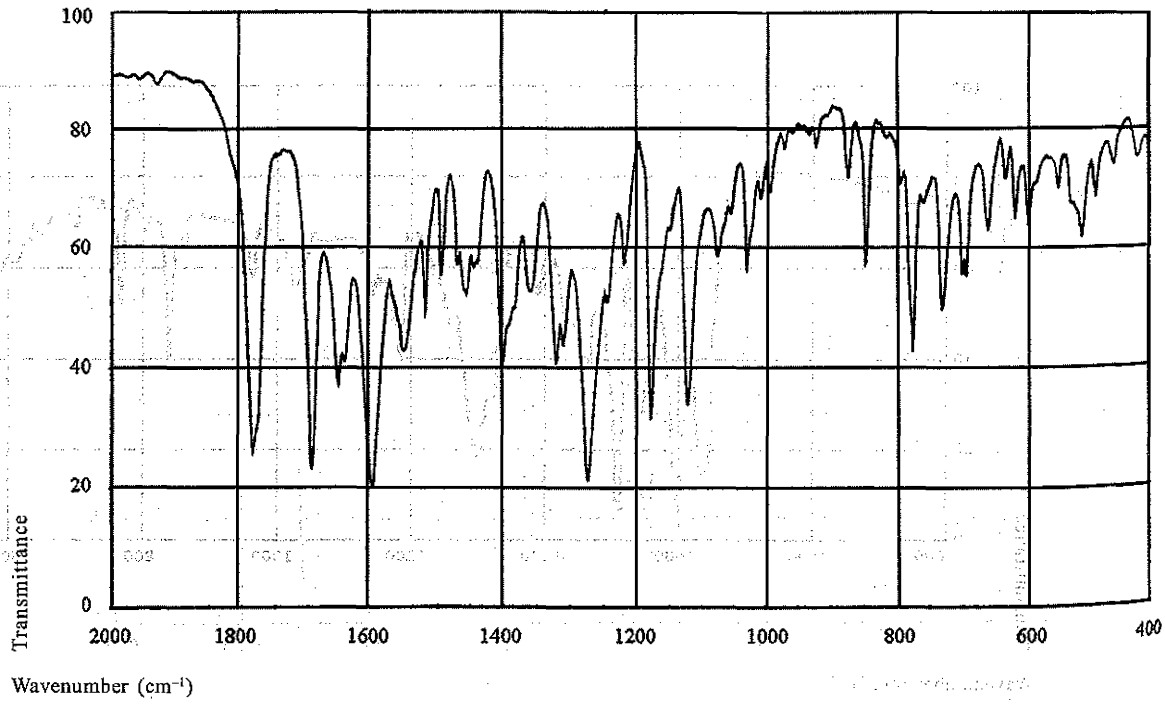
Phase: KBr disc



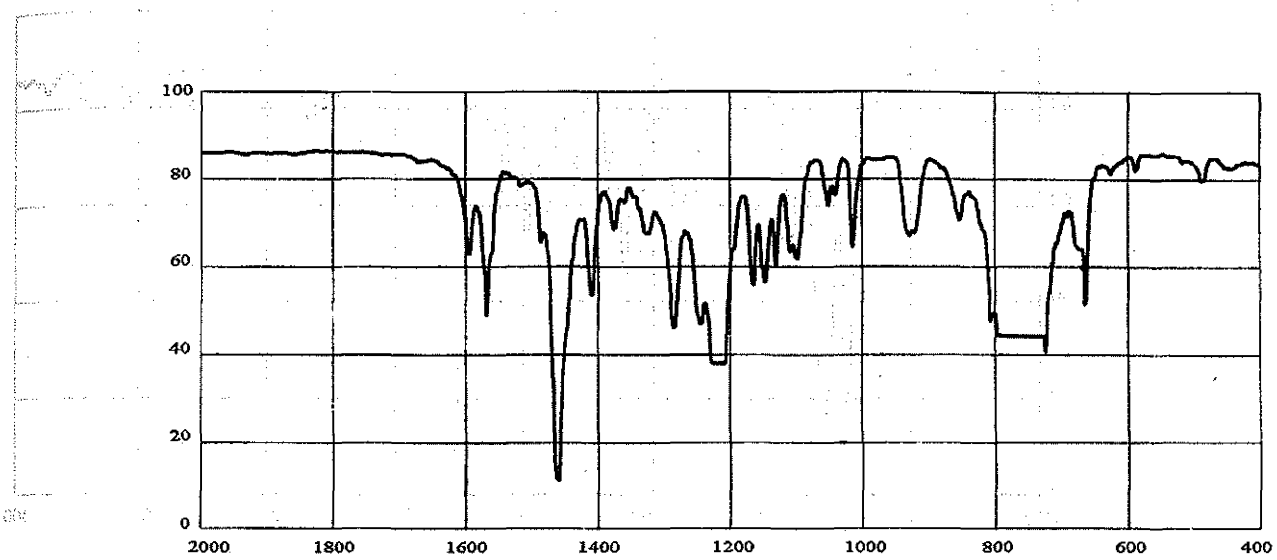
Procaine Penicillin

FTIR

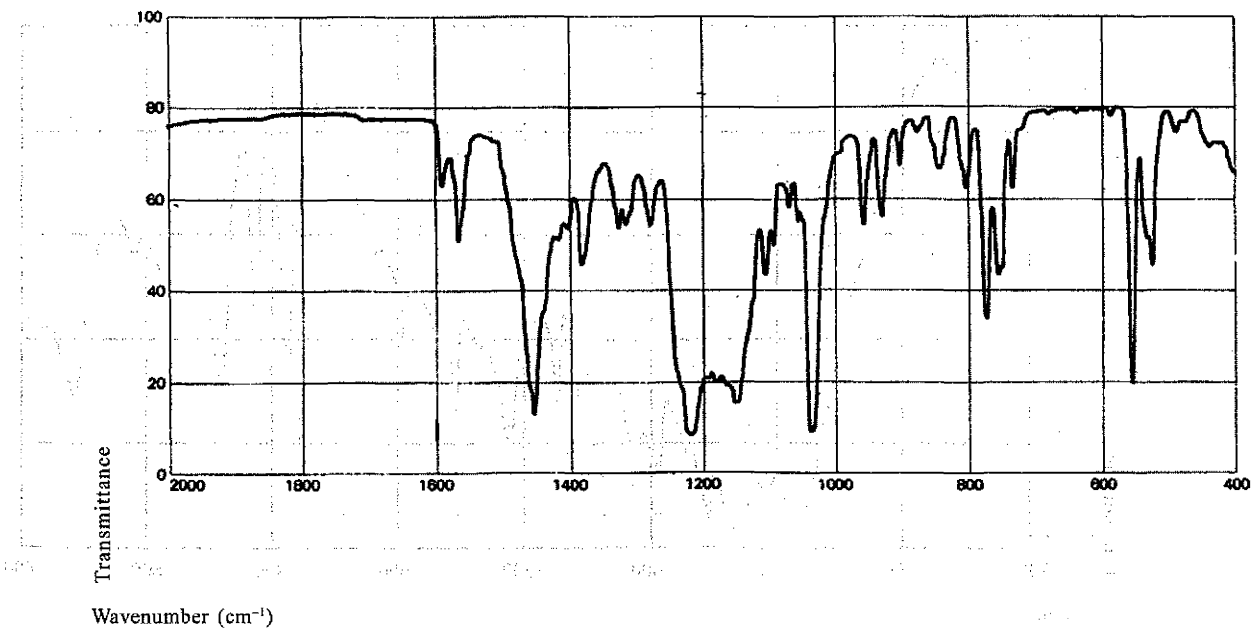
Phase: KBr disc



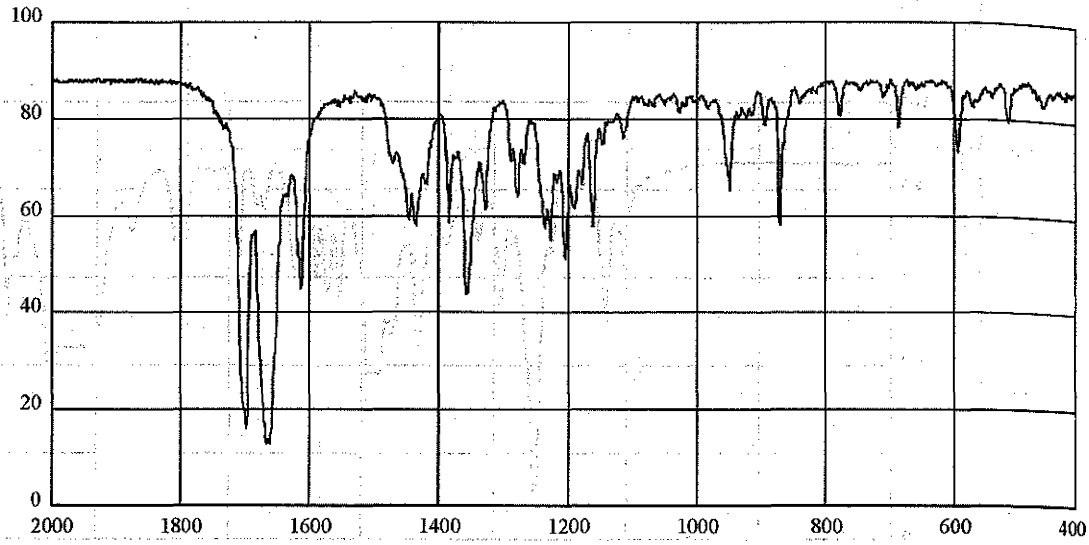
Prochlorperazine *Dispersive IR* Phase: 10 per cent solution in $CHCl_3$ Thickness 0.1 mm



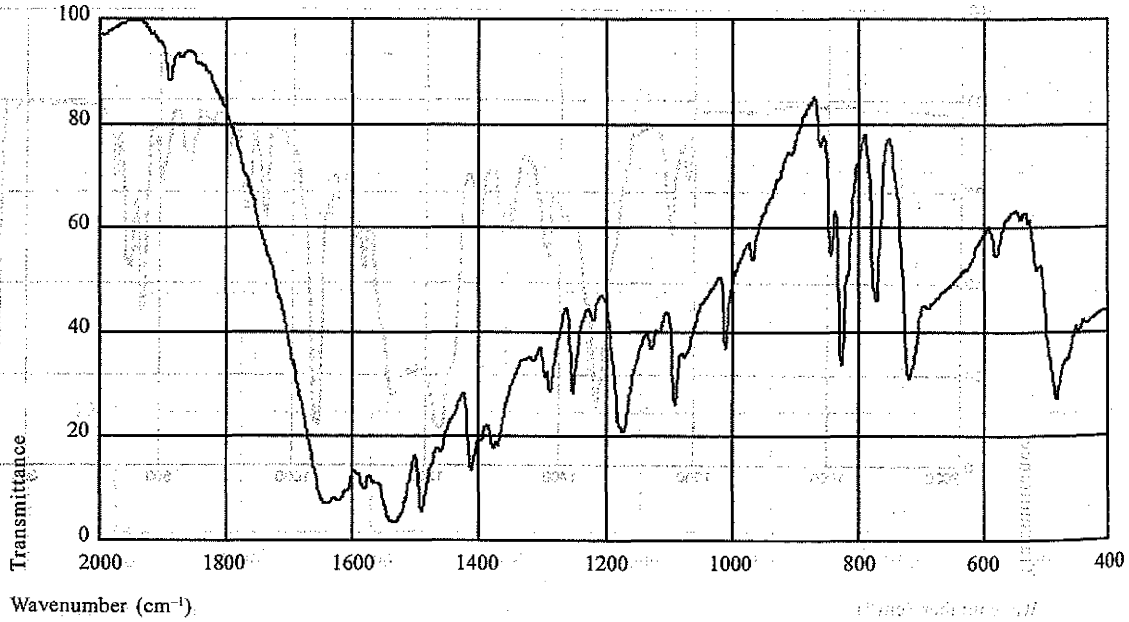
Prochlorperazine Mesylate *Dispersive IR* Phase: Liquid paraffin mull



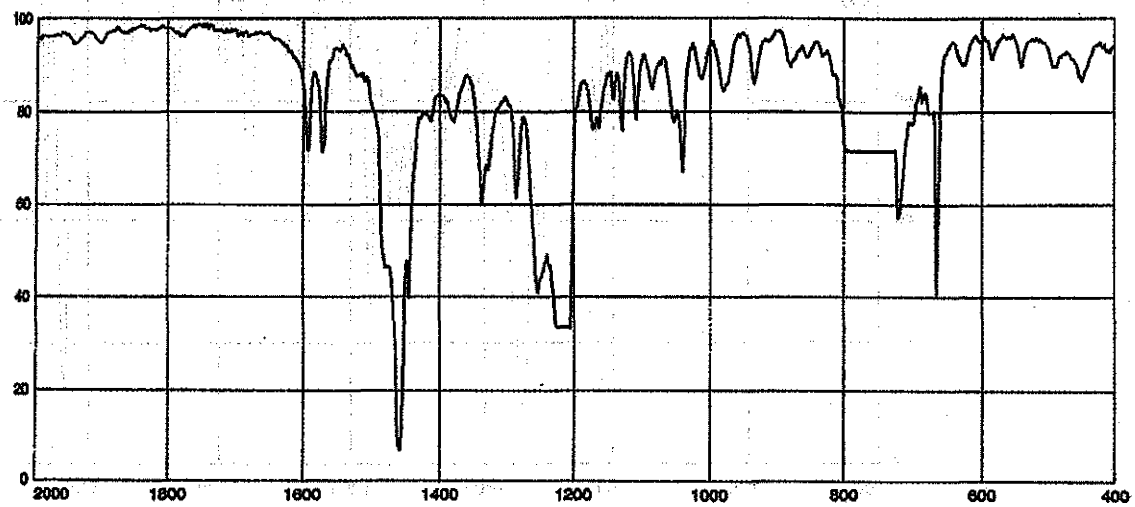
Progesterone *FTIR* Phase: KBr disc



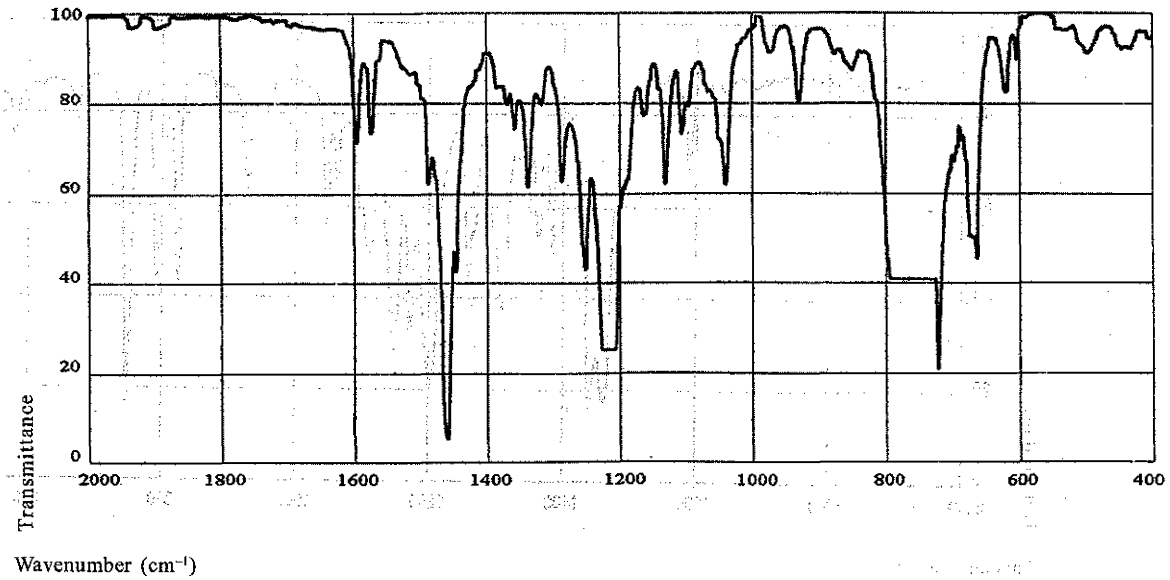
Proguanil Hydrochloride *FTIR* Phase: KCl disc



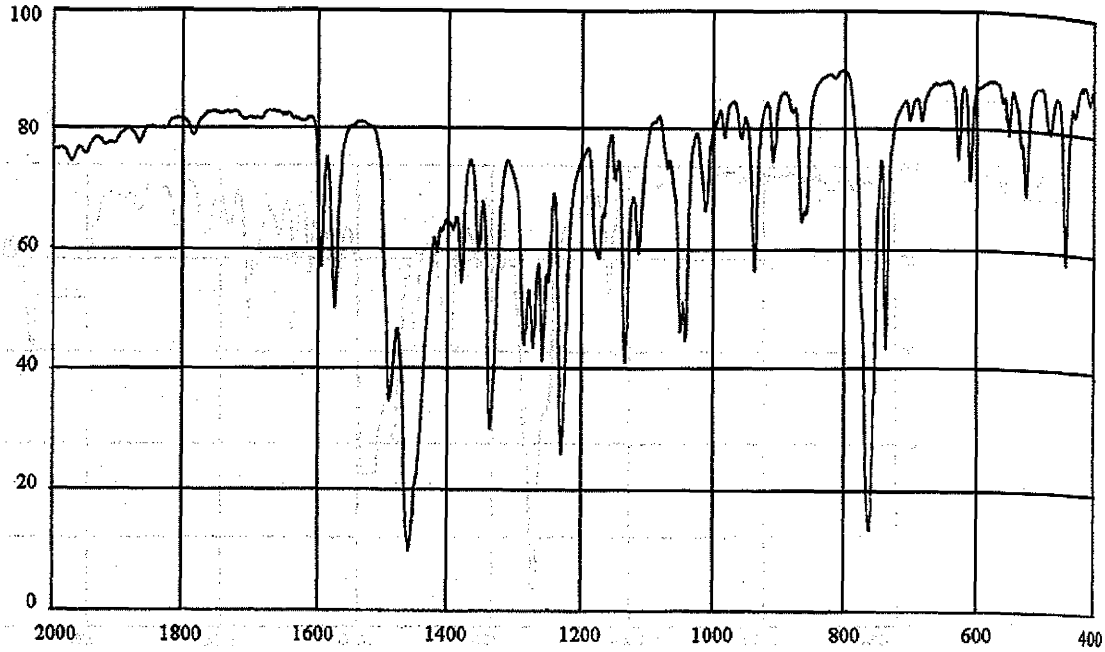
Promazine *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



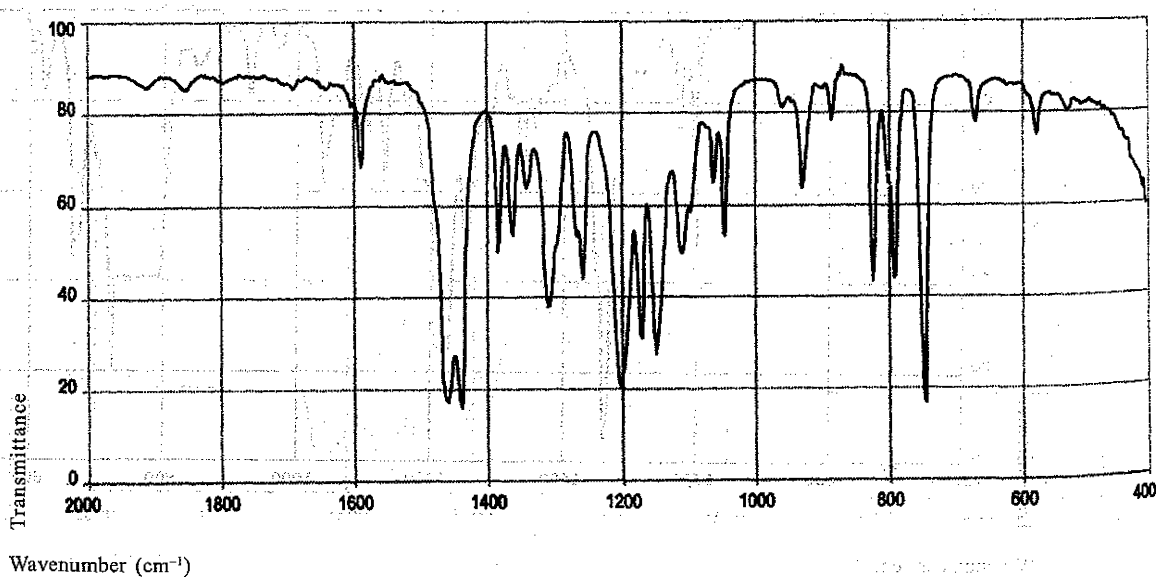
Promethazine *Dispersive IR* Phase: 5 per cent w/v solution in $CHCl_3$ Thickness 0.1 mm



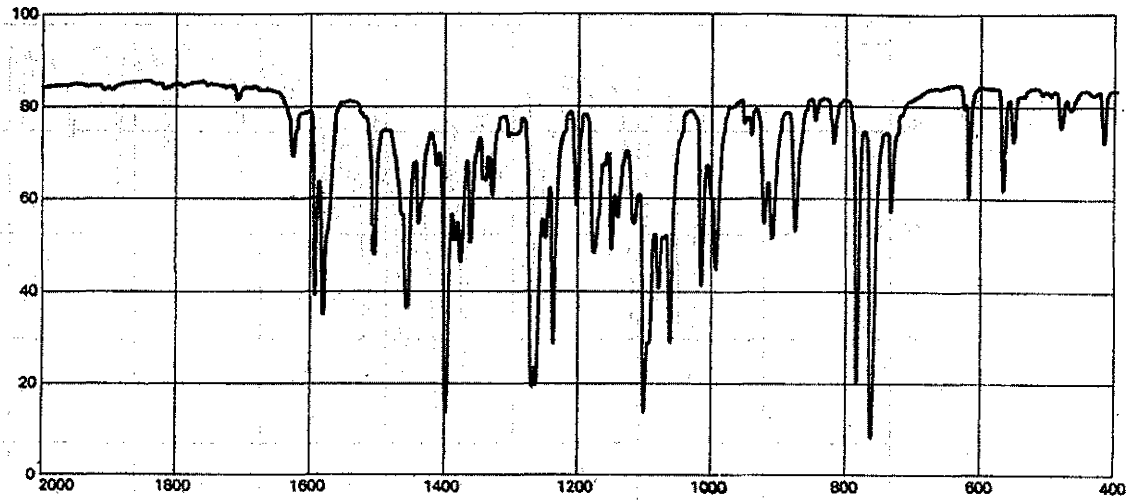
Promethazine Hydrochloride *FTIR* Phase: KBr disc



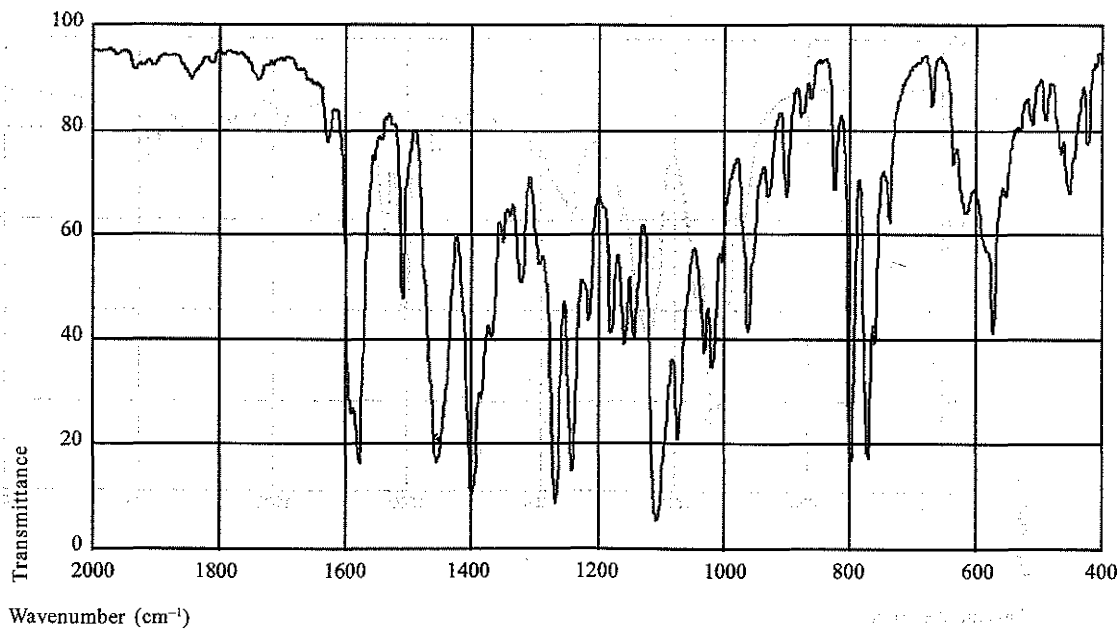
Propofol *FTIR* Phase: Thin film



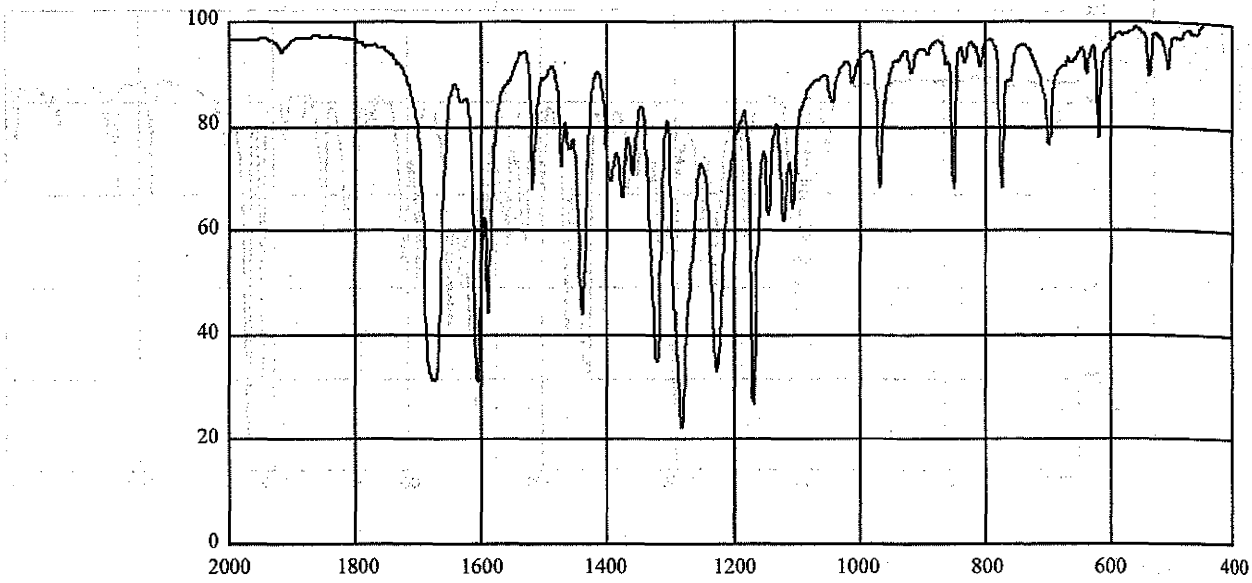
Propranolol *Dispersive IR* Phase: KBr disc



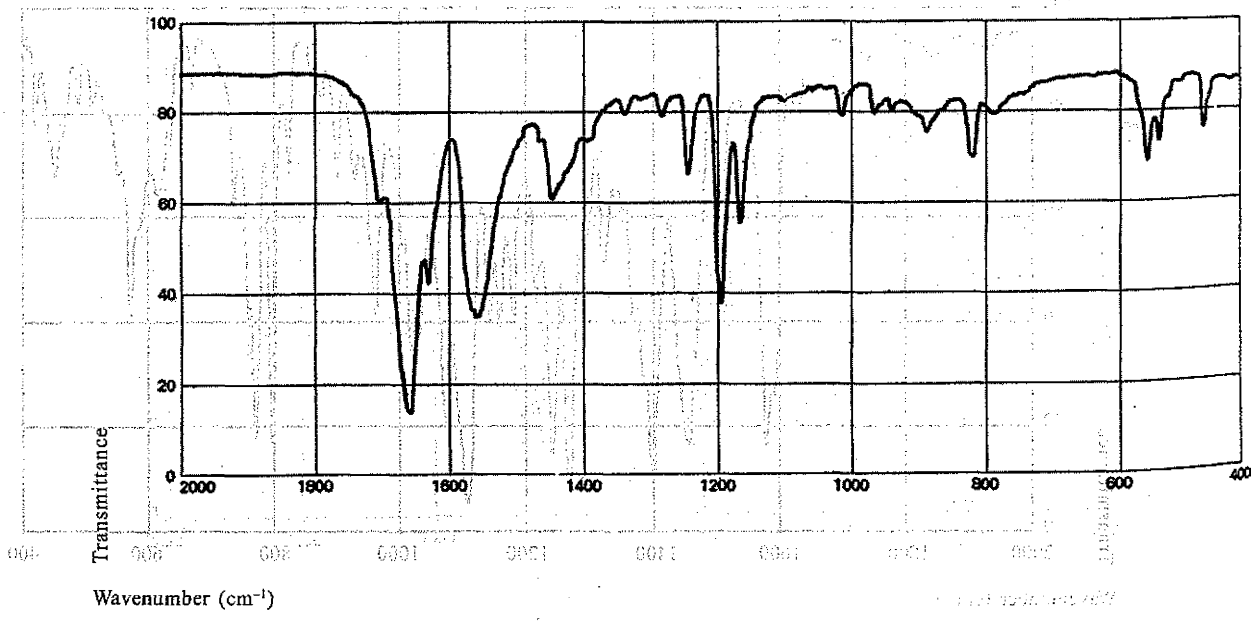
Propranolol Hydrochloride *FTIR* Phase: KBr disc



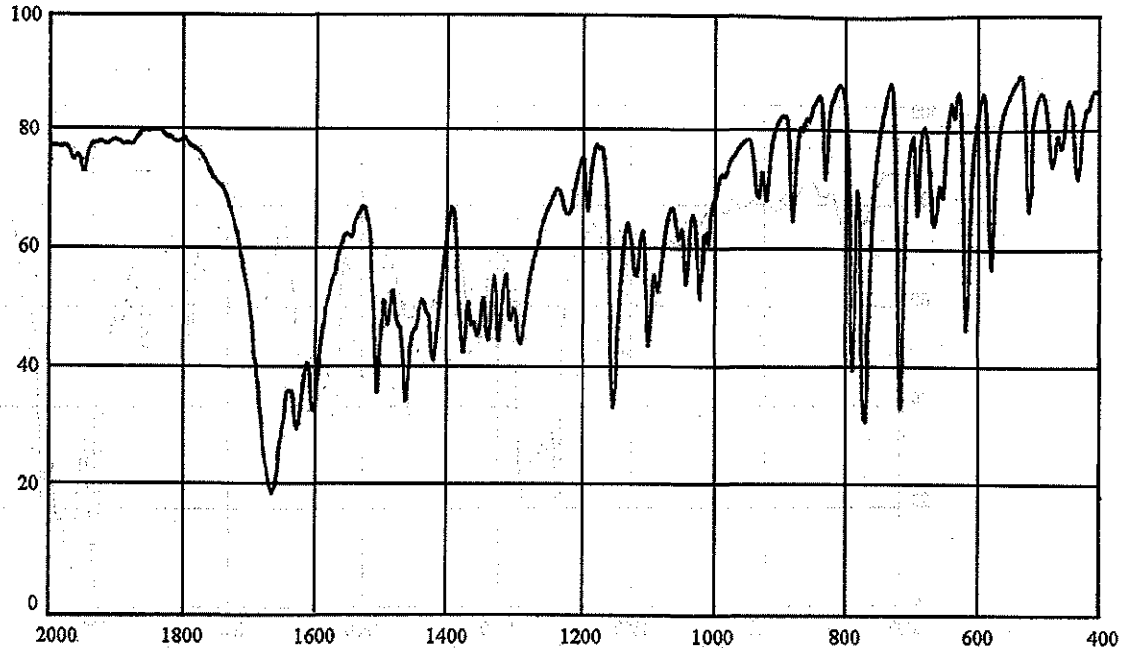
Propylparaben *FTIR* Phase: KBr disc



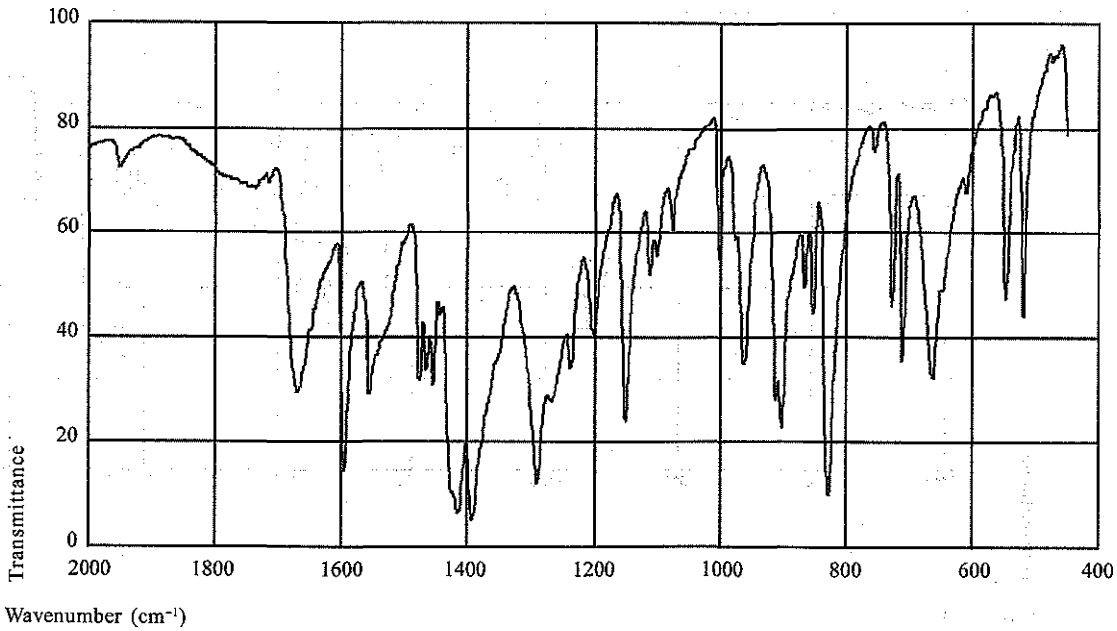
Propylthiouracil *Dispersive IR* Phase: KBr disc



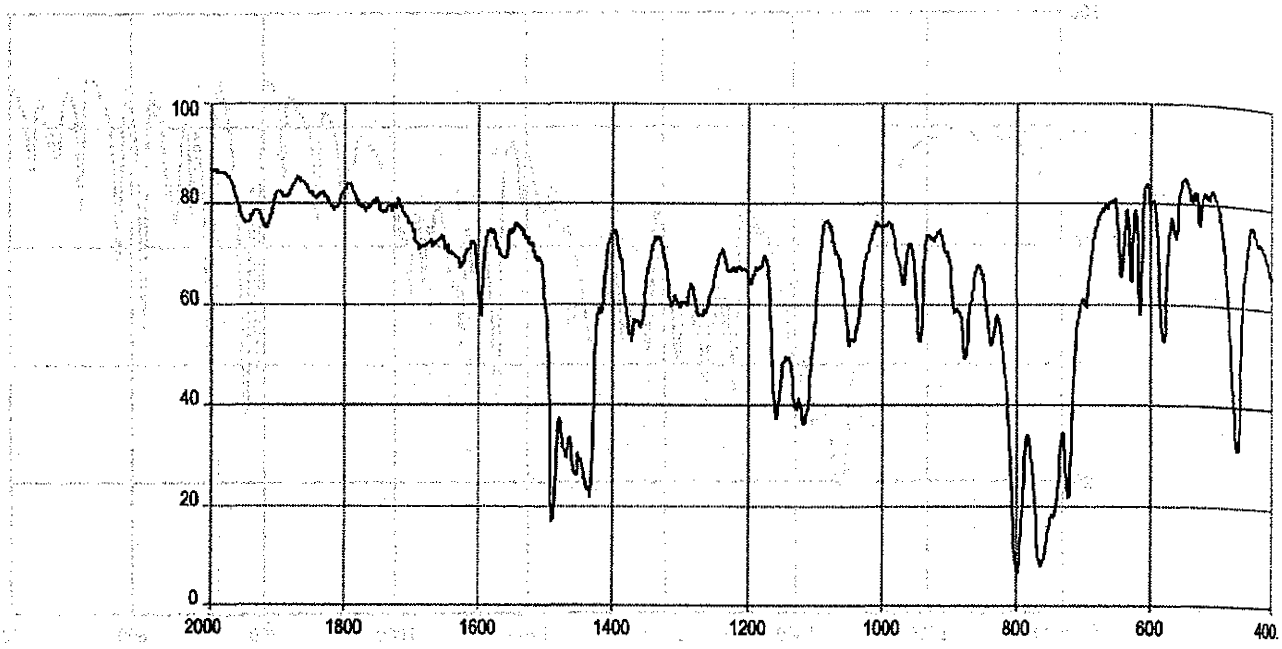
Propyphenazone *FTIR* Phase: KBr disc



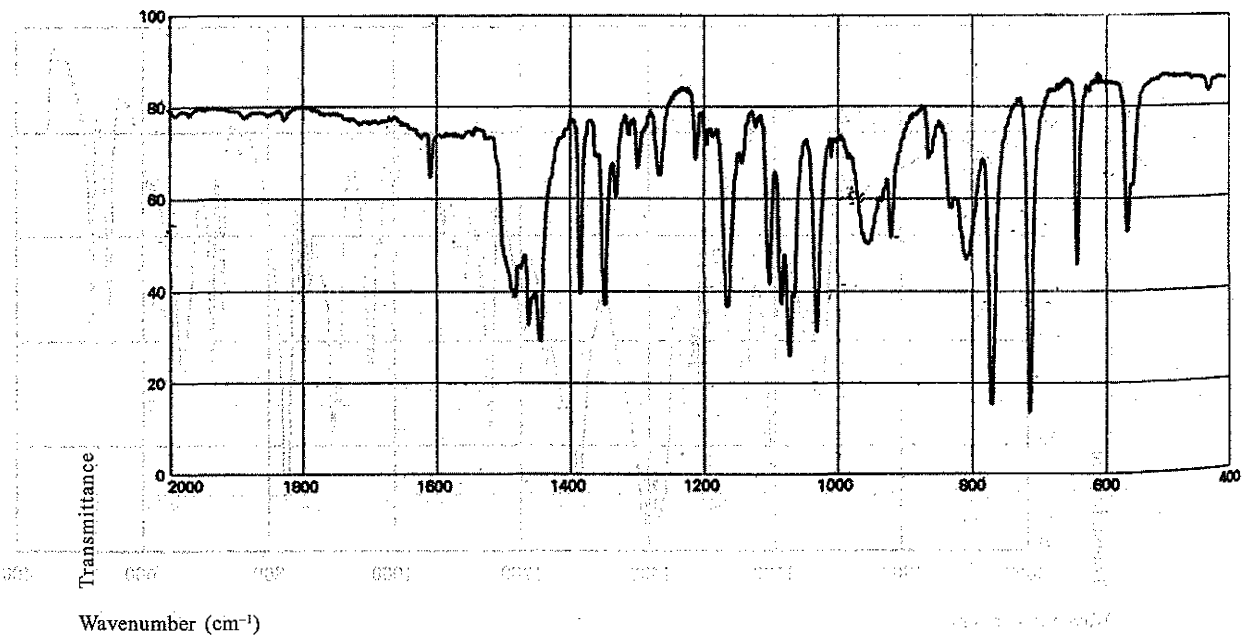
Prothionamide *FTIR* Phase: KBr disc



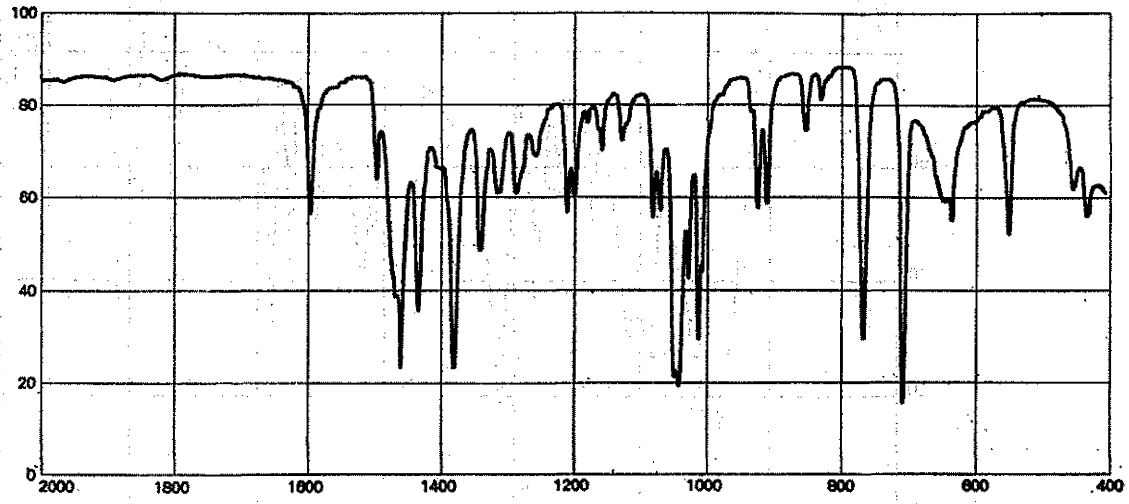
Protriptyline *Dispersive IR* Phase: Thin film



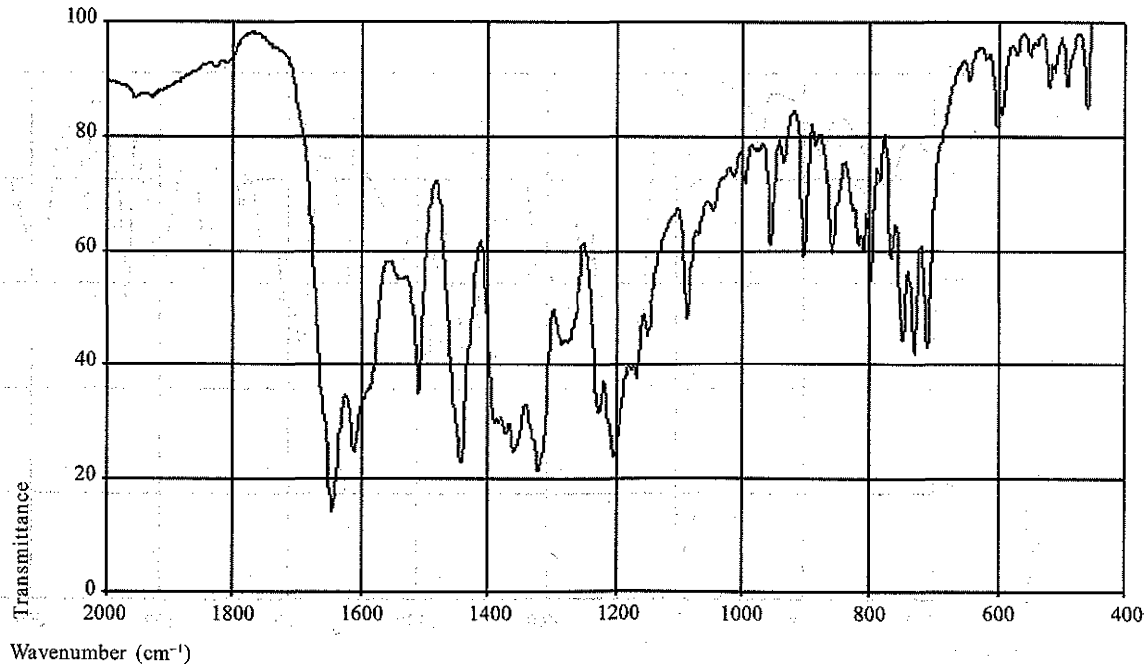
Pseudoephedrine *Dispersive IR* Phase: KBr disc



Pseudoephedrine Hydrochloride *Dispersive IR* Phase: KCl disc

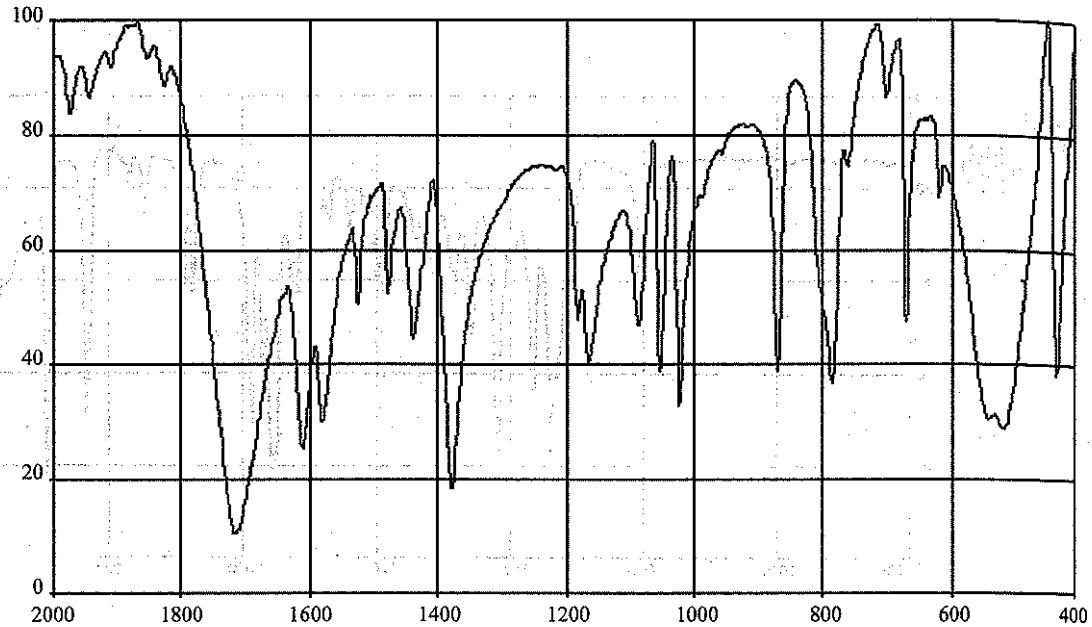


Pyrantel Pamoate *FTIR* Phase: KBr disc



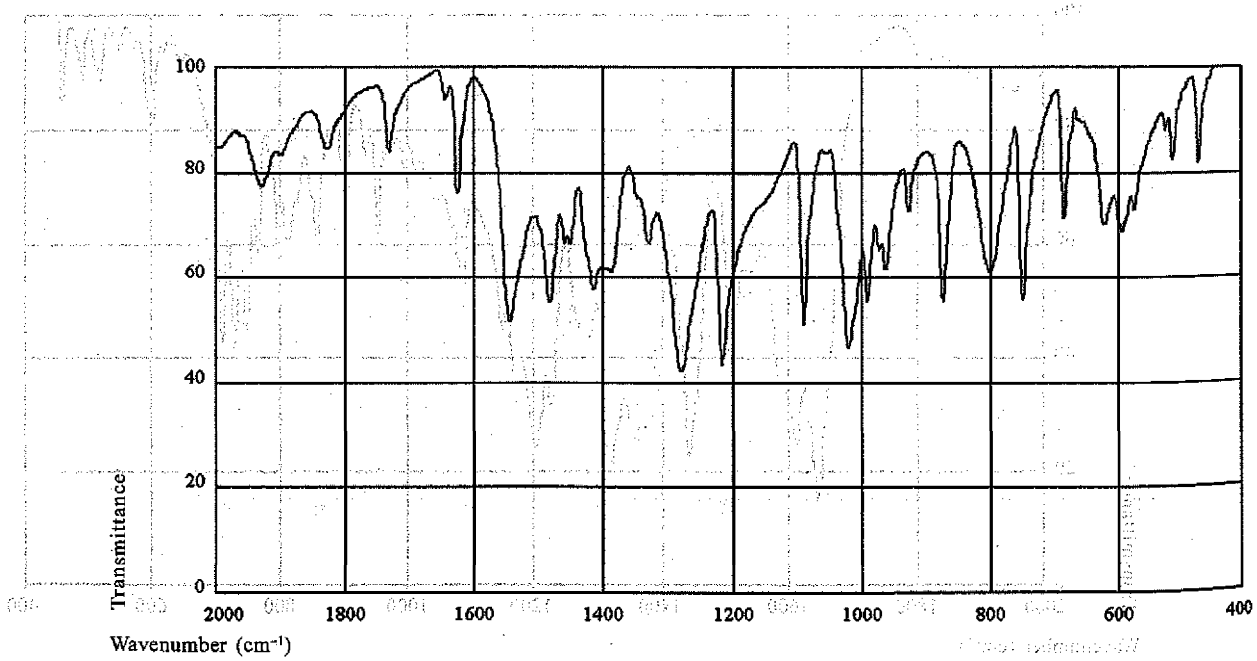
Pyrazinamide

FTIR Phase: KBr disc

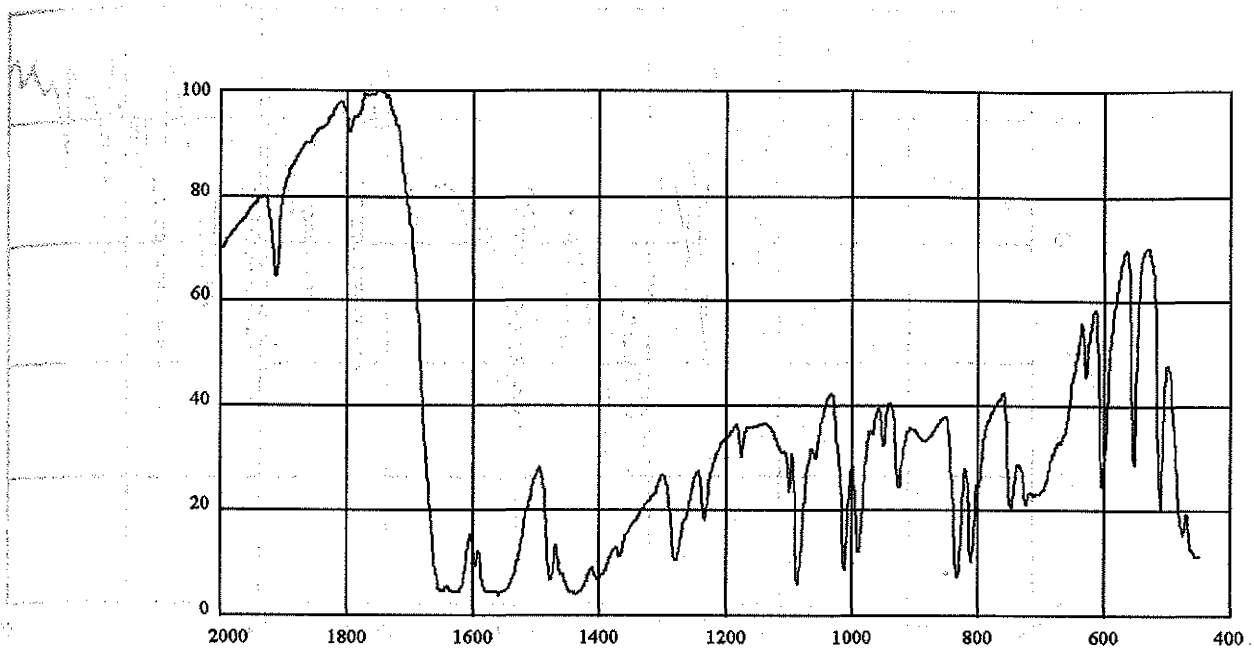


Pyridoxine Hydrochloride

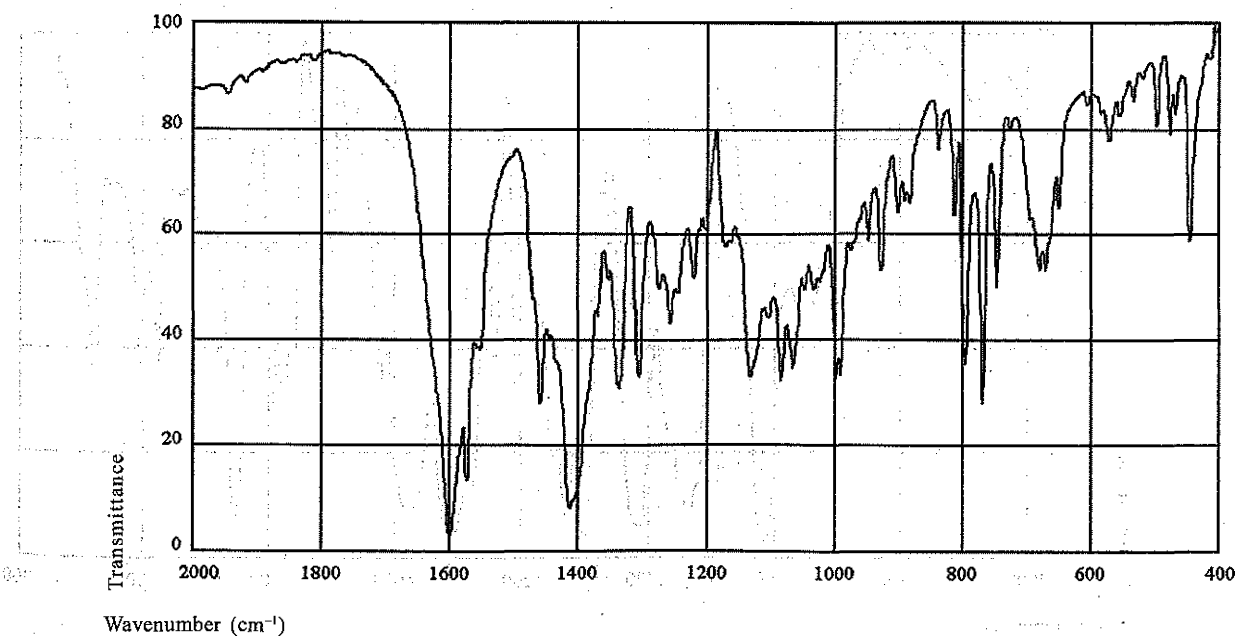
FTIR Phase: KBr disc



Pyrimethamine *FTIR* Phase: KBr disc



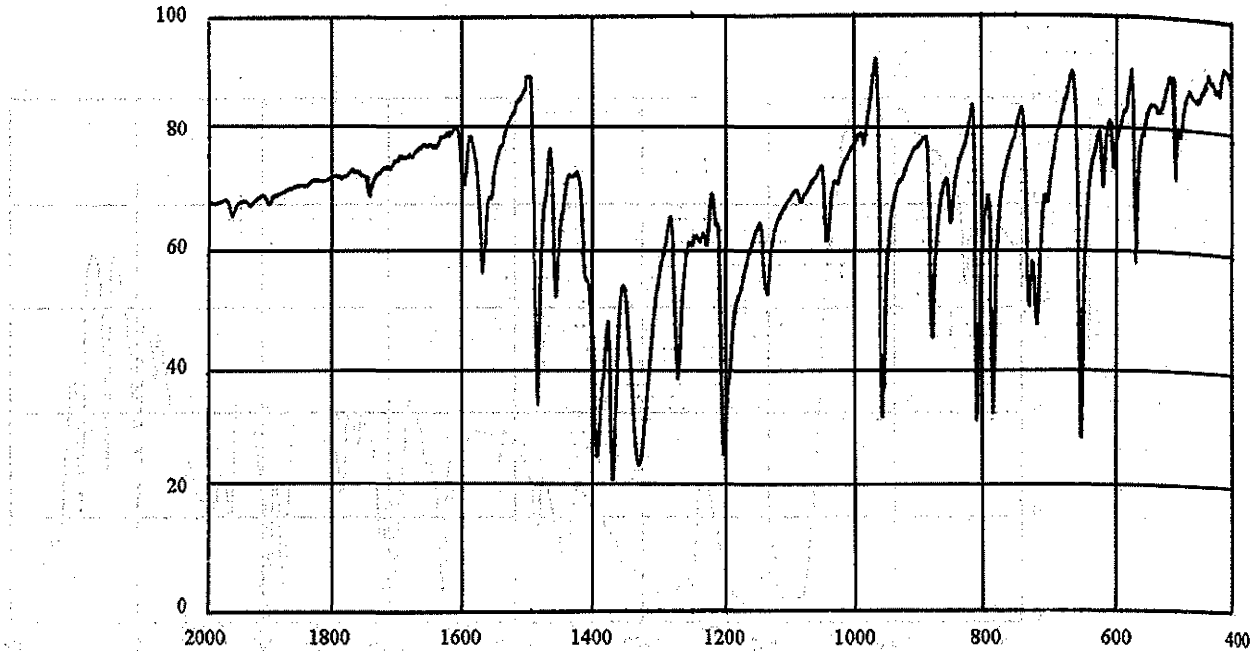
Quetiapine Fumarate *FTIR* Phase: KBr disc



Quiniodochlor

FTIR

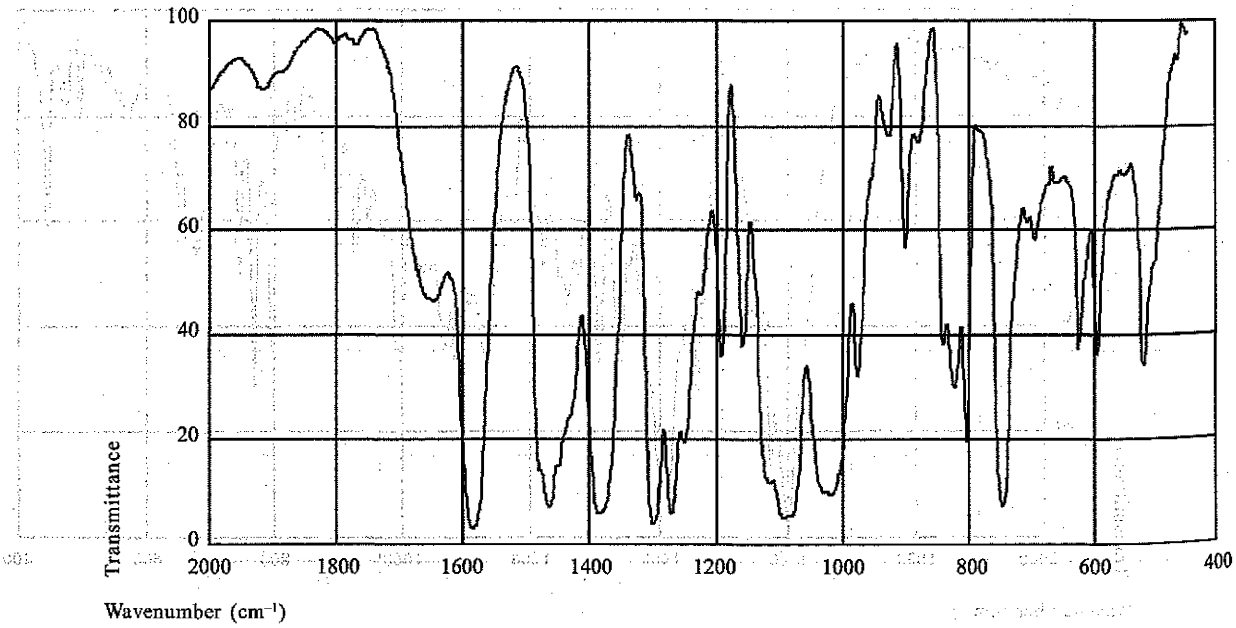
Phase: KBr disc



Rabeprazole Sodium

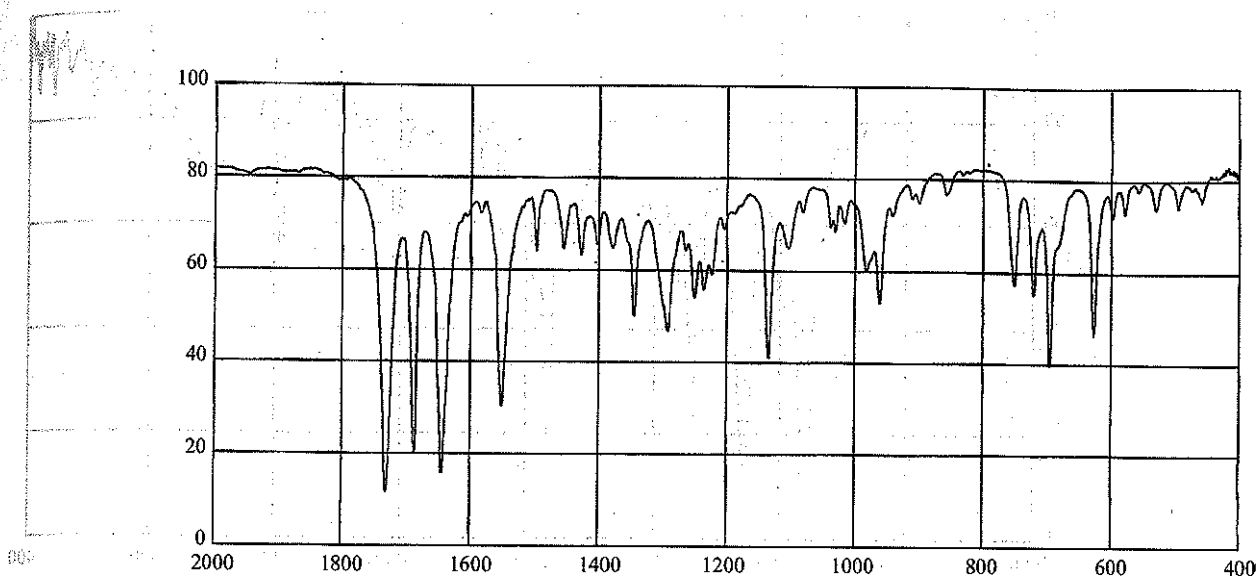
FTIR

Phase: KBr disc

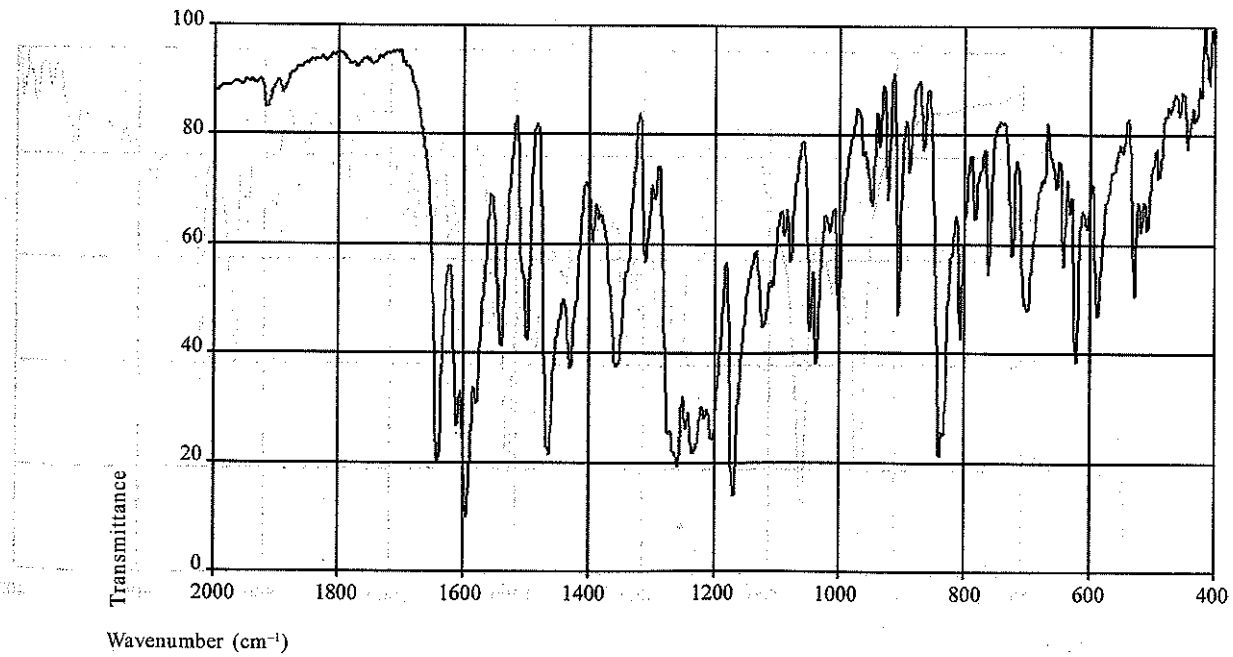


UNAUTHORISED COPY

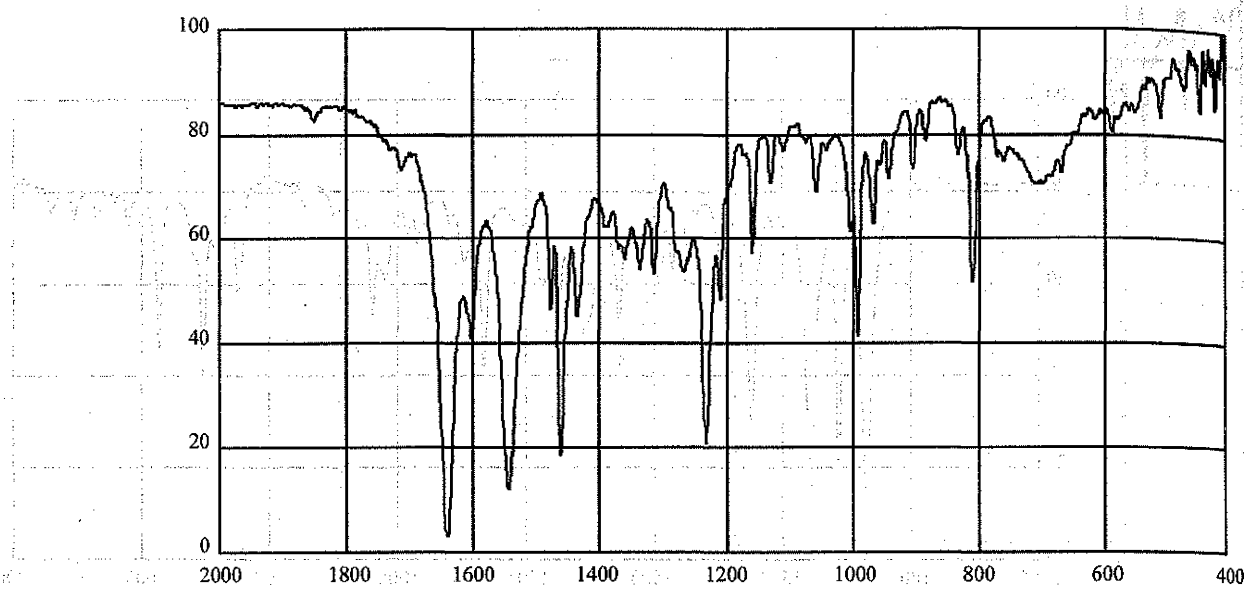
Racecadotril *FTIR* Phase: KBr disc



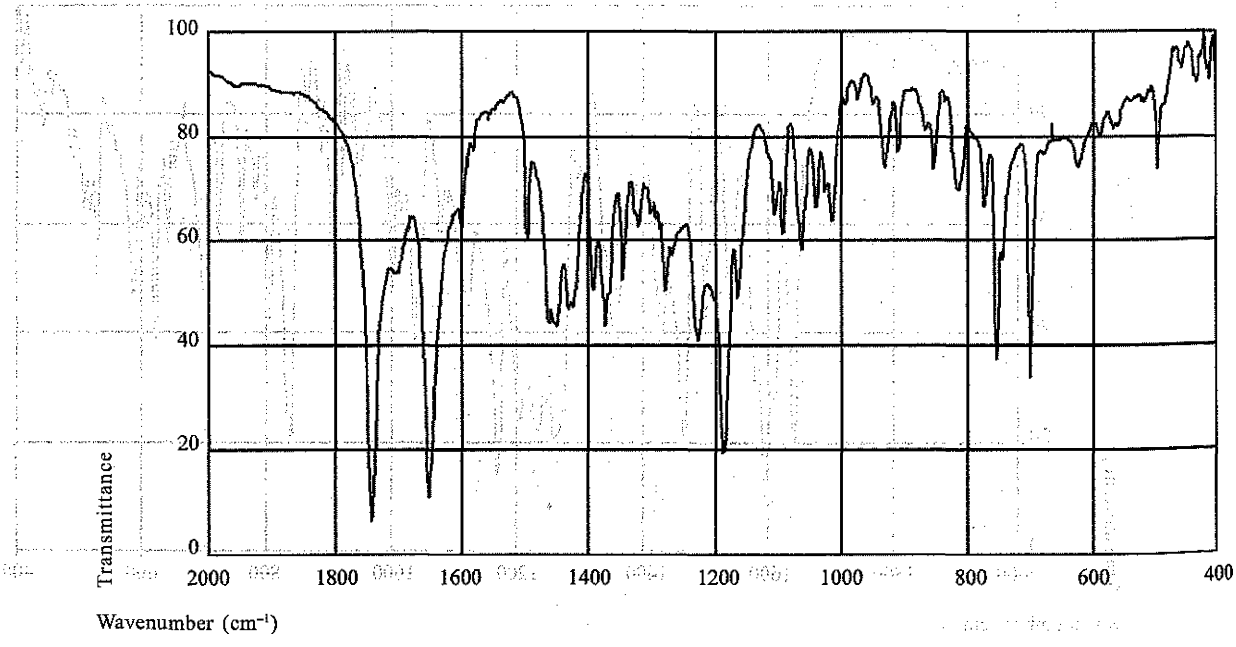
Raloxifene Hydrochloride *FTIR* Phase: KBr disc



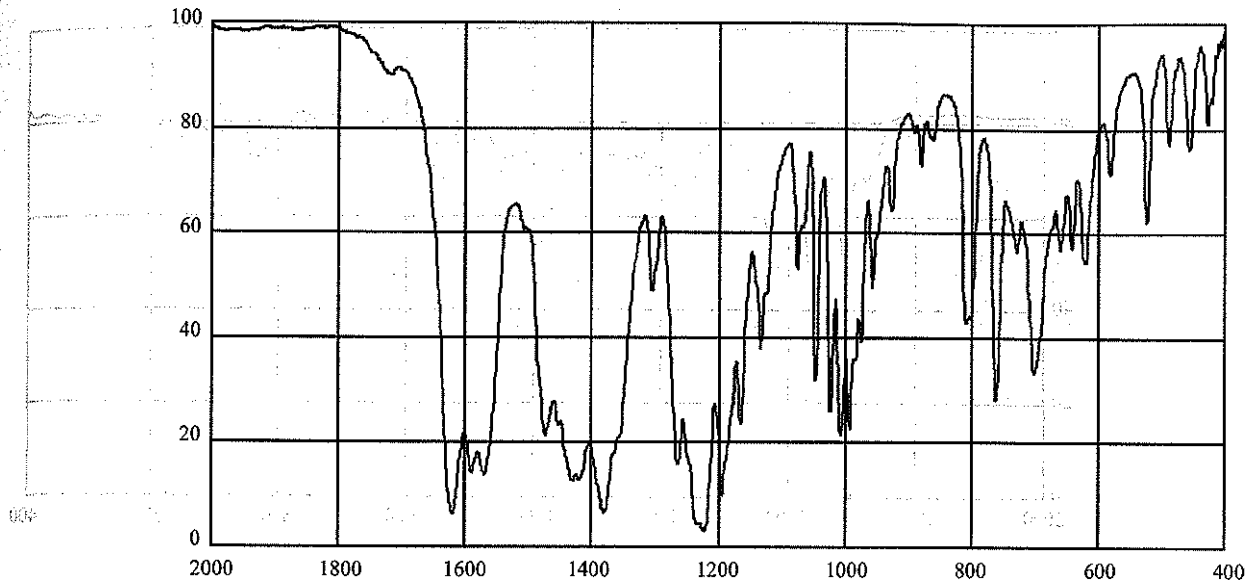
Ramelteon *FTIR* Phase: KBr disc



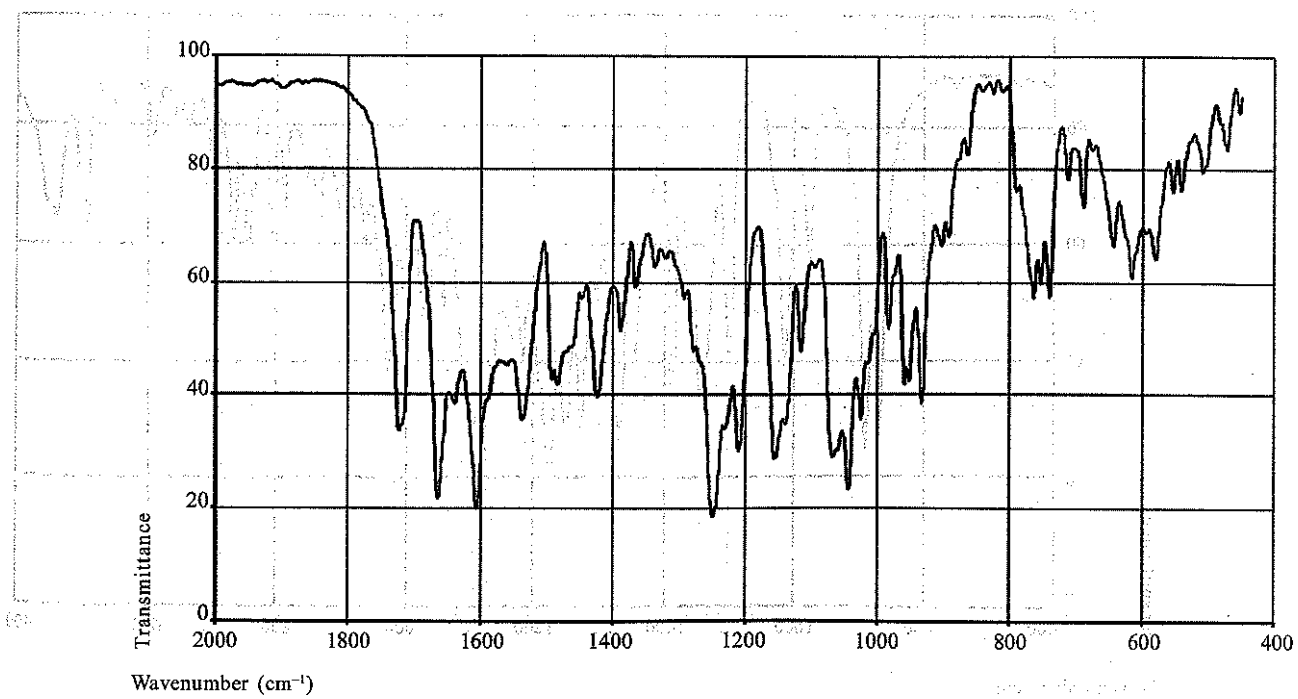
Ramipril *FTIR* Phase: KBr disc



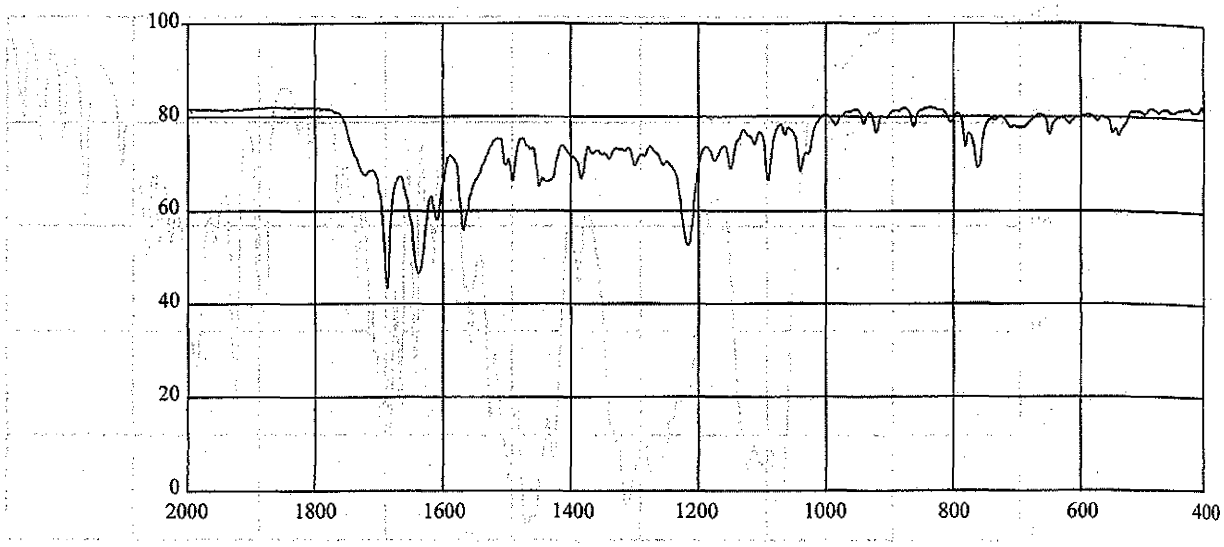
Ranitidine Hydrochloride *FTIR* Phase: KCl disc



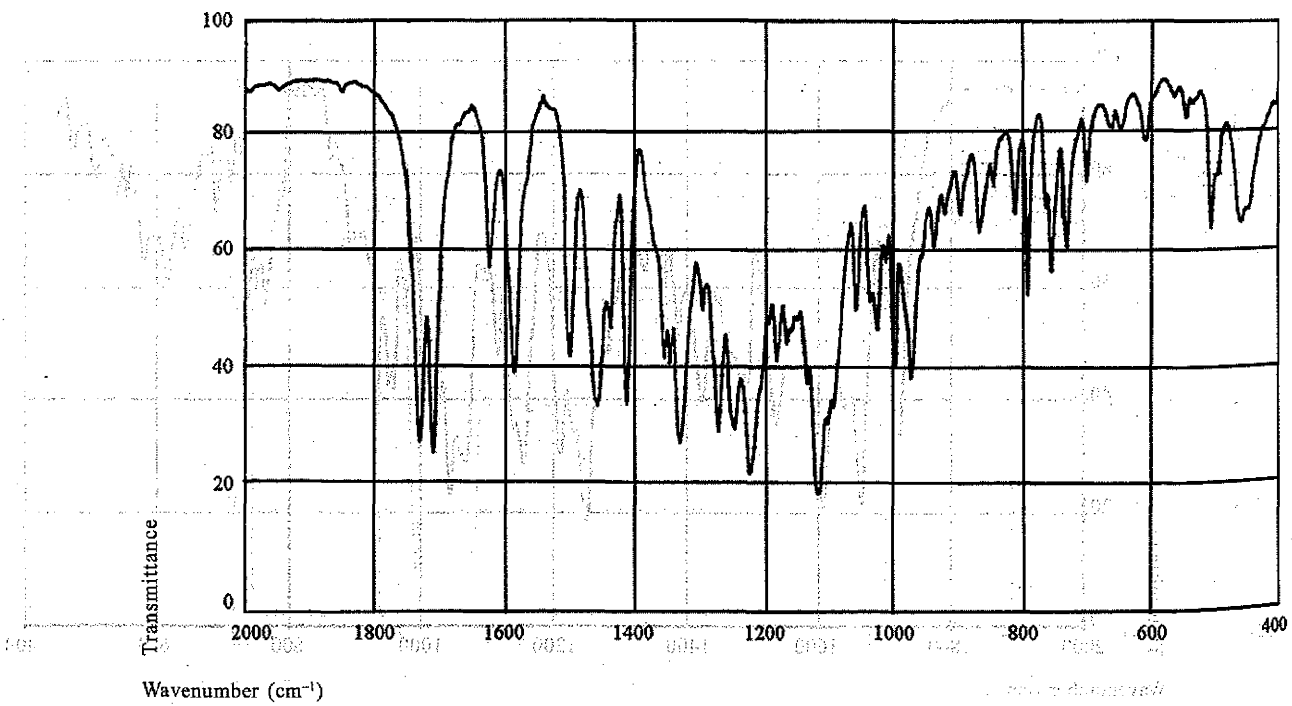
Remdesivir *FTIR* Phase: KBr disc



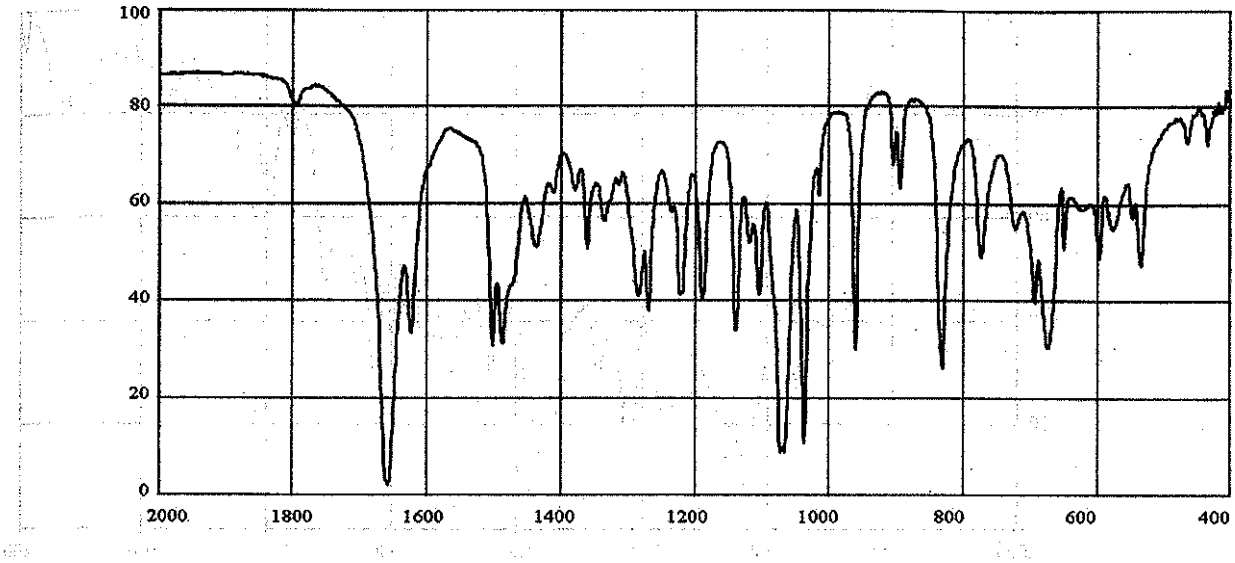
Repaglinide *FTIR* Phase: KBr disc



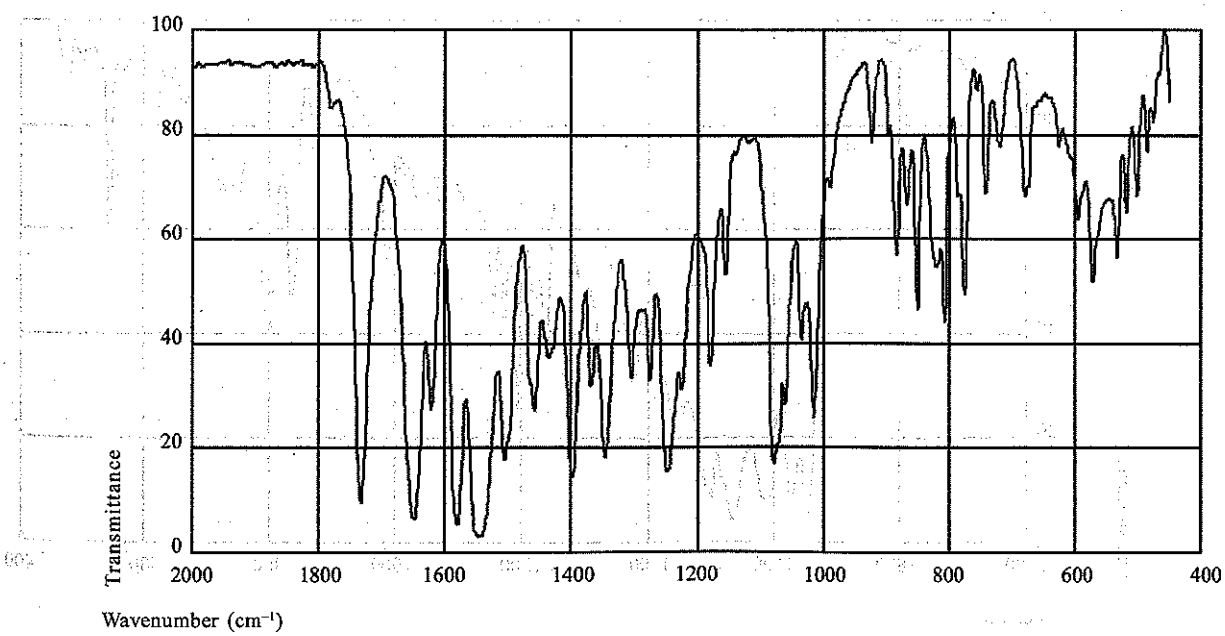
Reserpine *FTIR* Phase: KBr disc



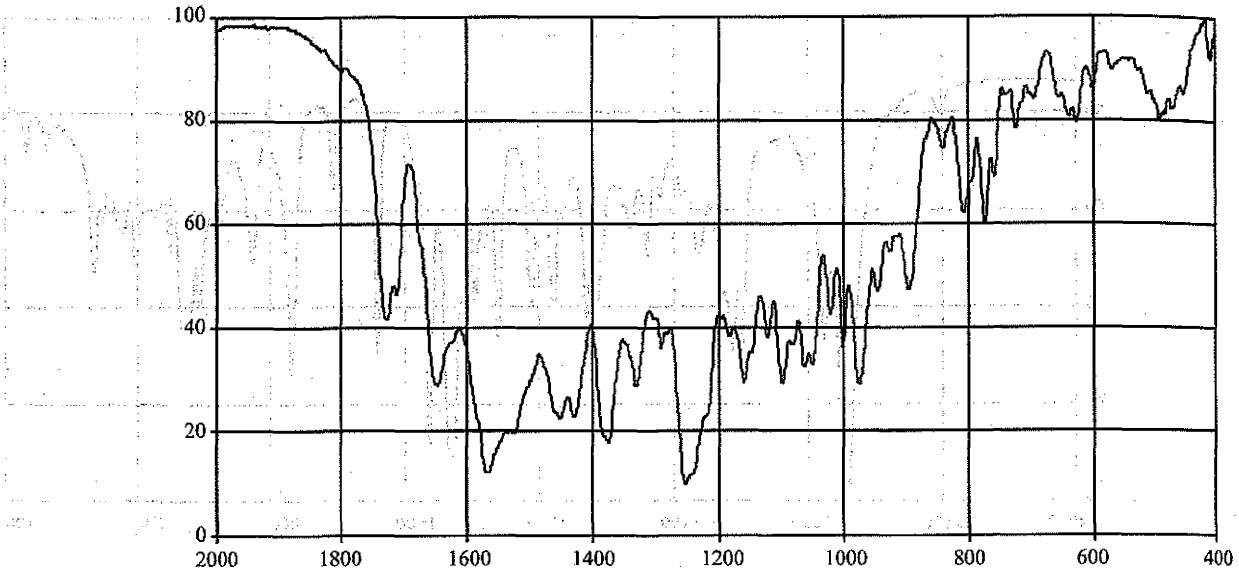
Ribavirin *FTIR* Phase: KBr disc



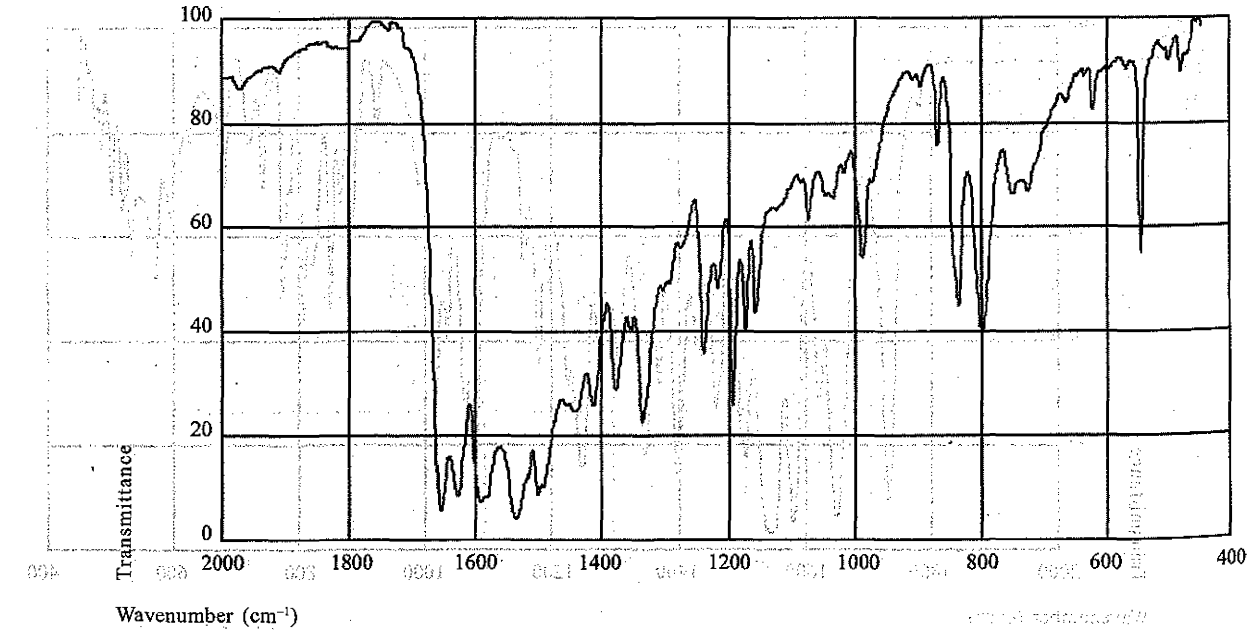
Riboflavin *FTIR* Phase: KBr disc



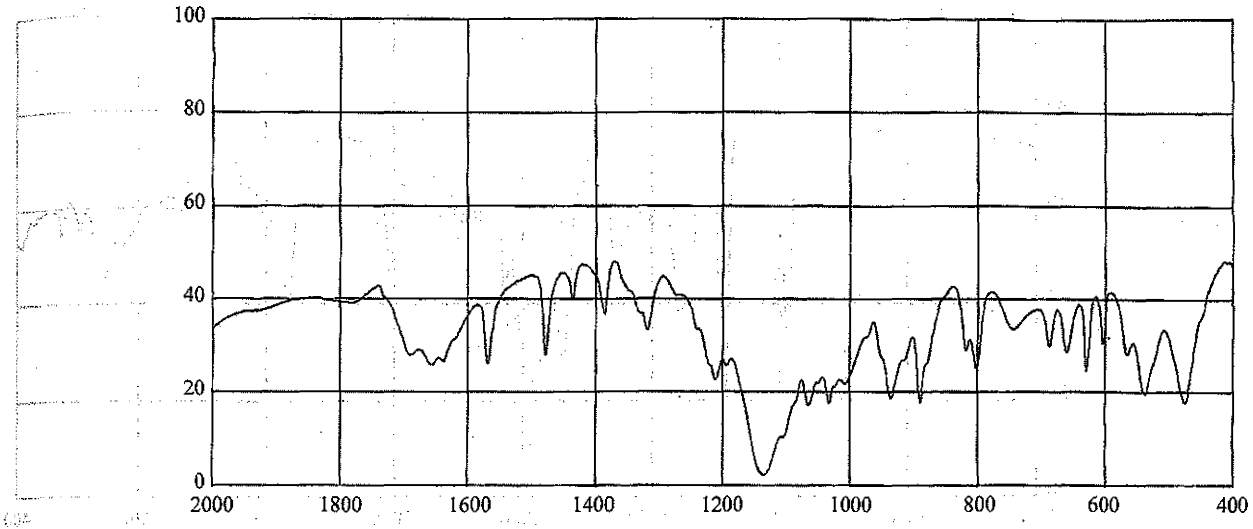
Rifampicin *FTIR* Phase: KBr disc



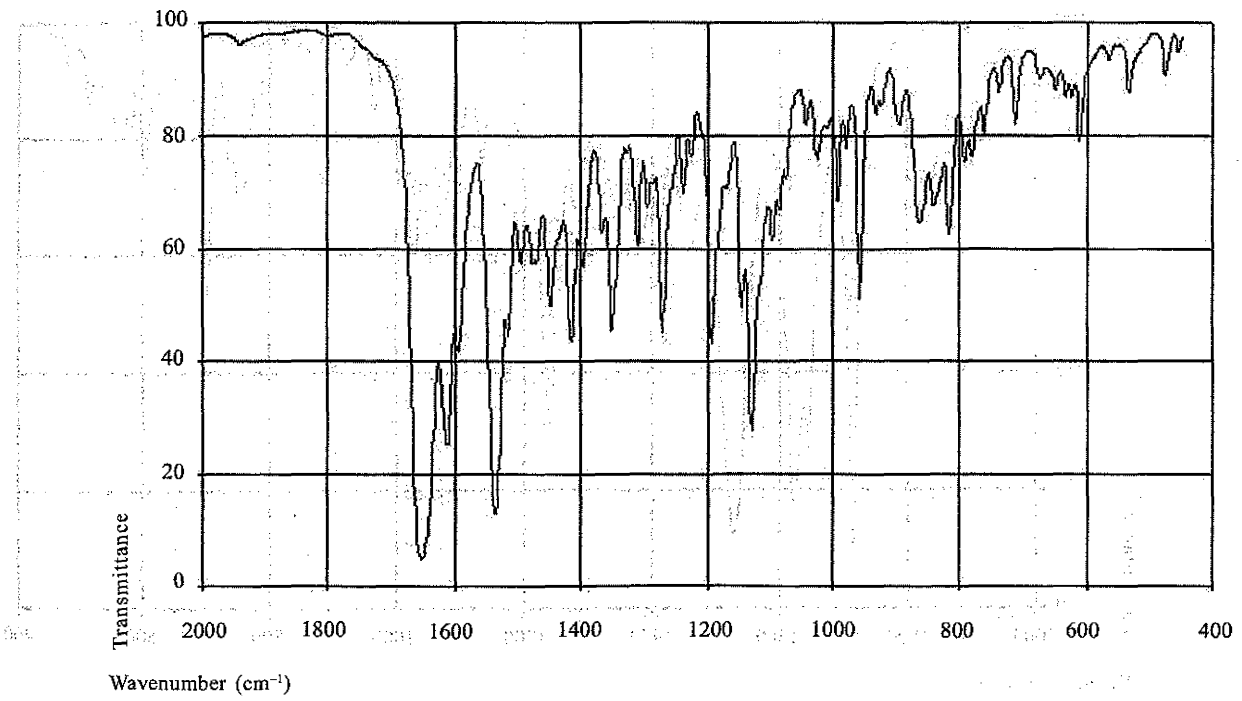
Rilpivirine *FTIR* Phase: KBr disc



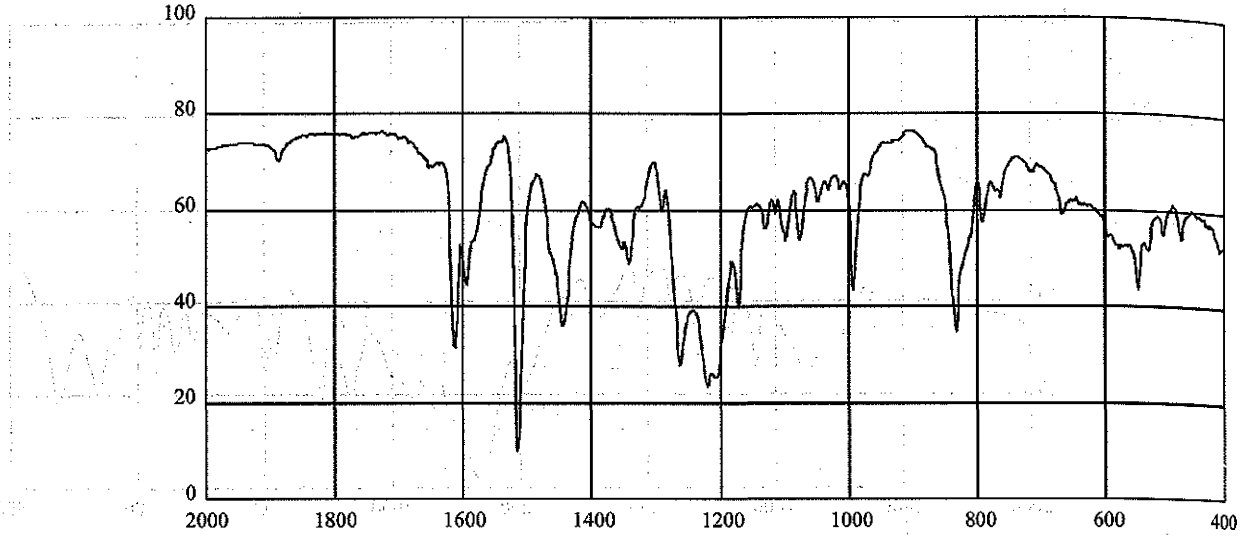
Risedronate Sodium *FTIR* Phase: KBr disc



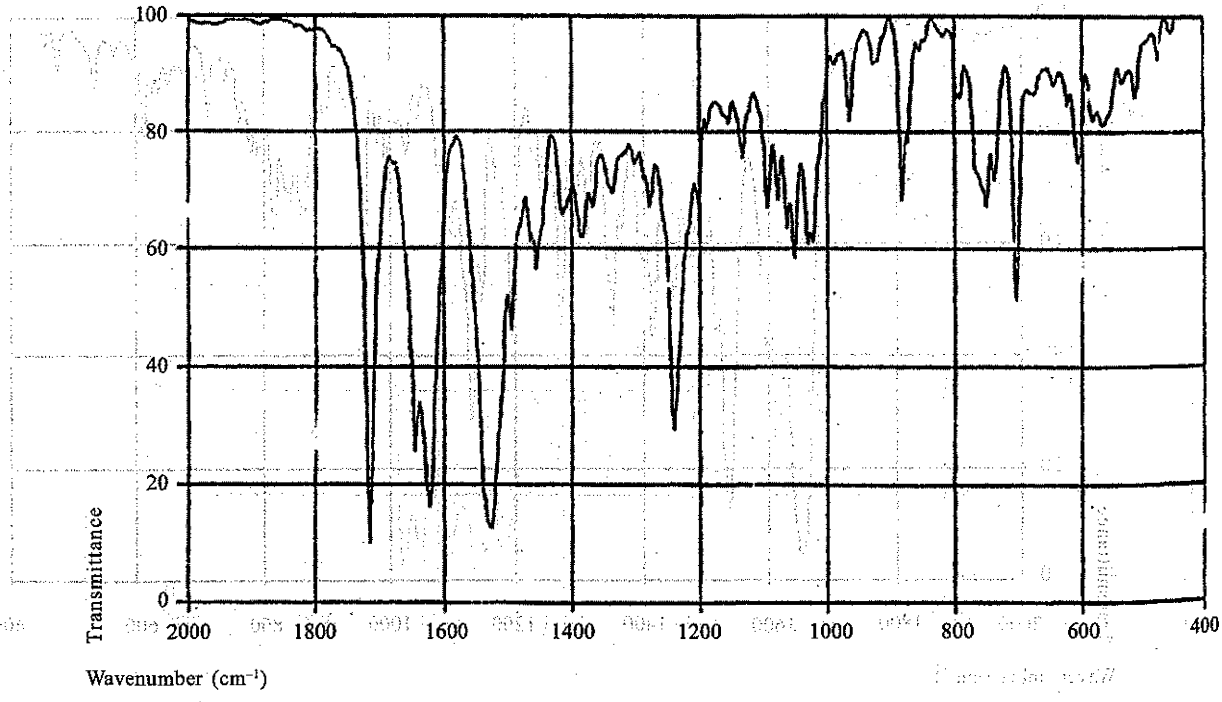
Risperidone *FTIR* Phase: KBr disc



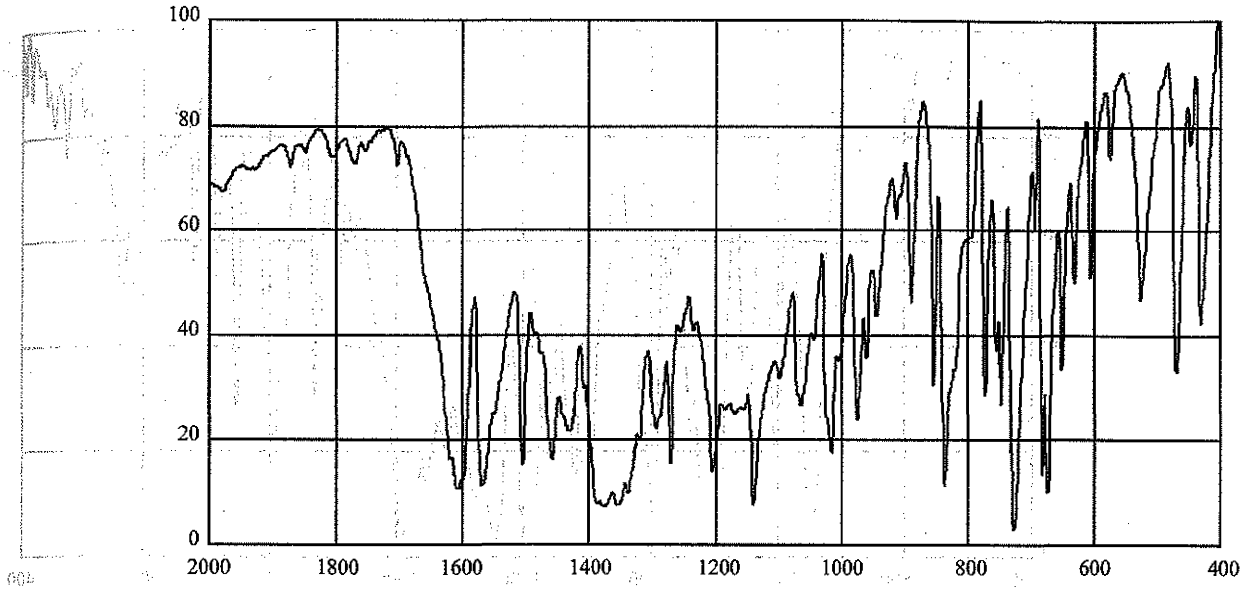
Ritodrine Hydrochloride *FTIR* Phase: KBr disc



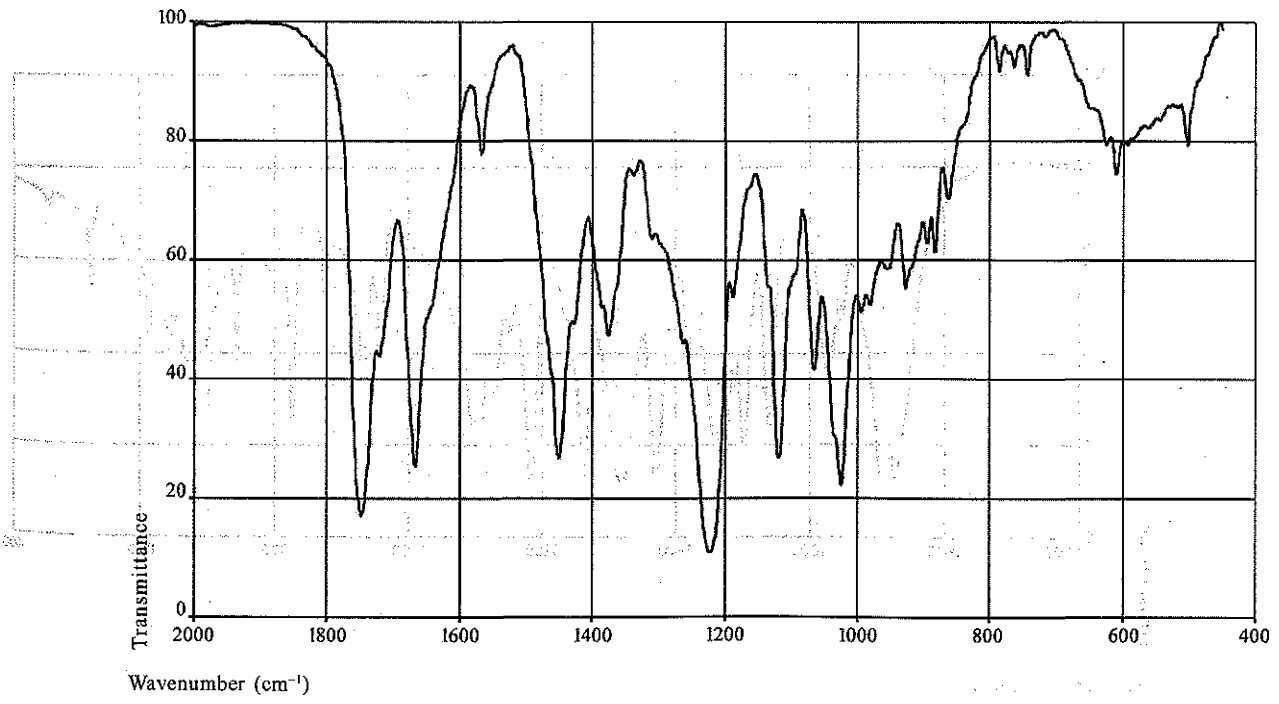
Ritonavir *FTIR* Phase: KBr disc



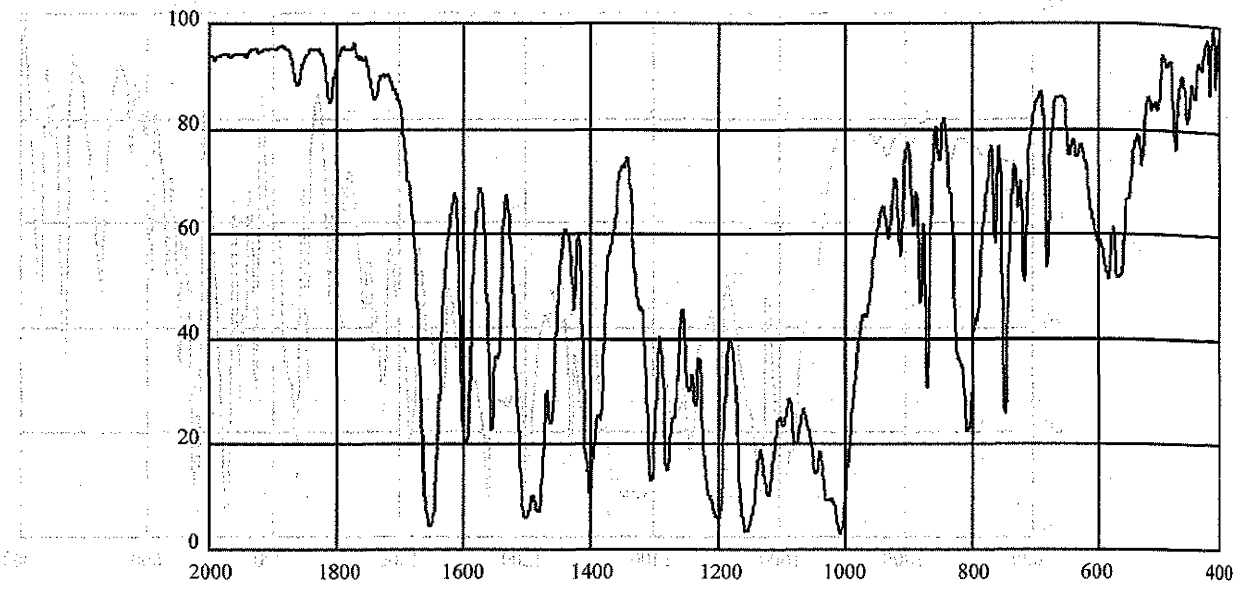
Rizatriptan Benzoate *FTIR* Phase: KBr disc



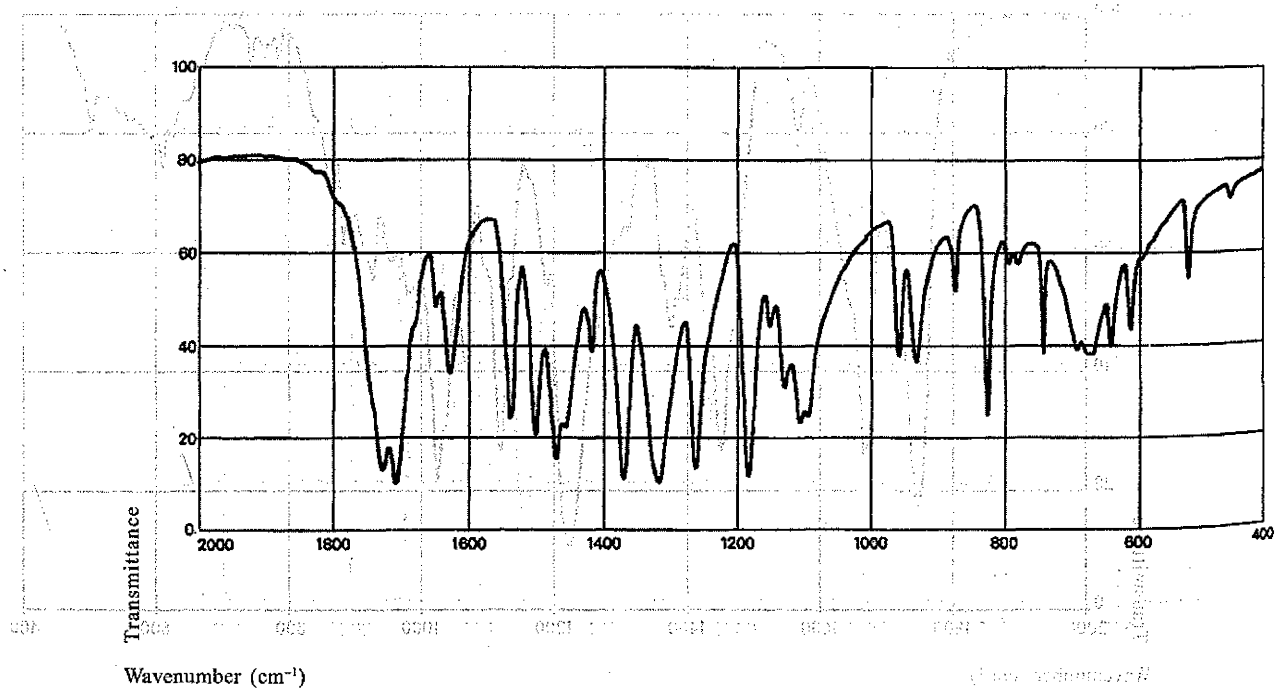
Rocuronium Bromide *FTIR* Phase: KBr disc



Roflumilast *FTIR* Phase: KBr disc



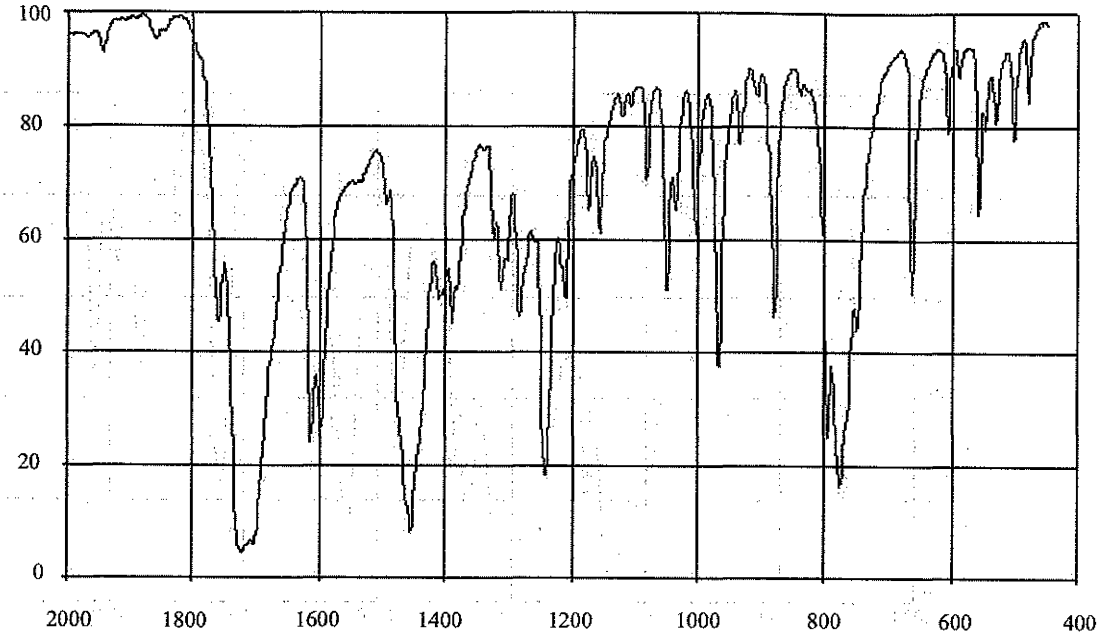
Ronidazole *Dispersive IR* Phase: KBr disc



Ropinirole Hydrochloride

FTIR

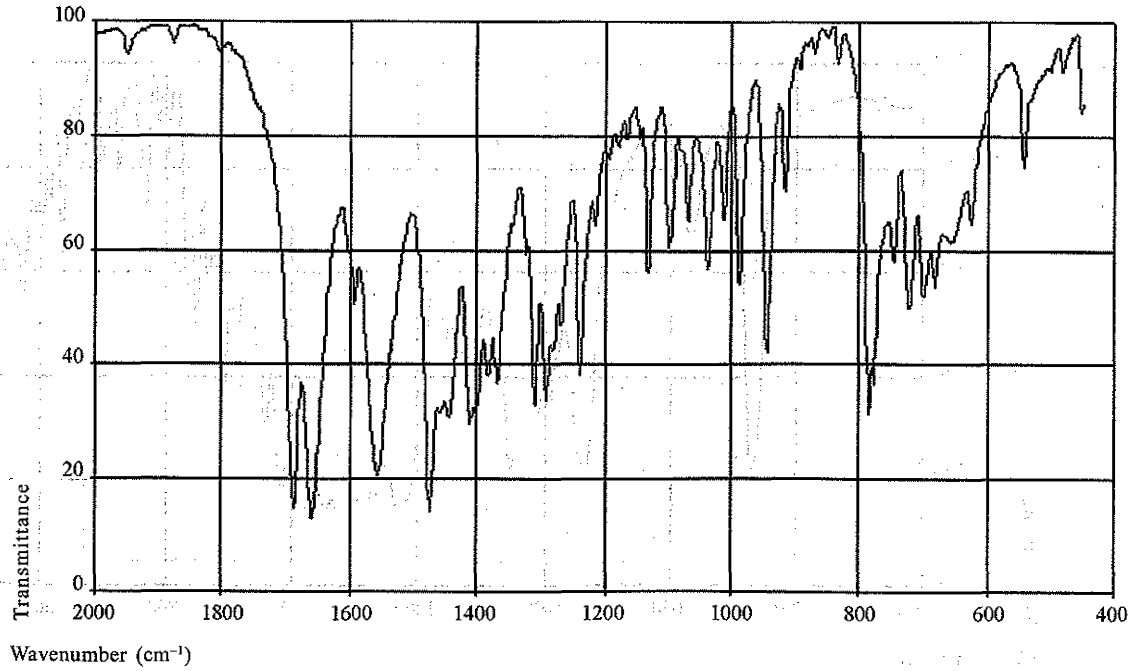
Phase: KBr disc



Ropivacaine Hydrochloride

FTIR

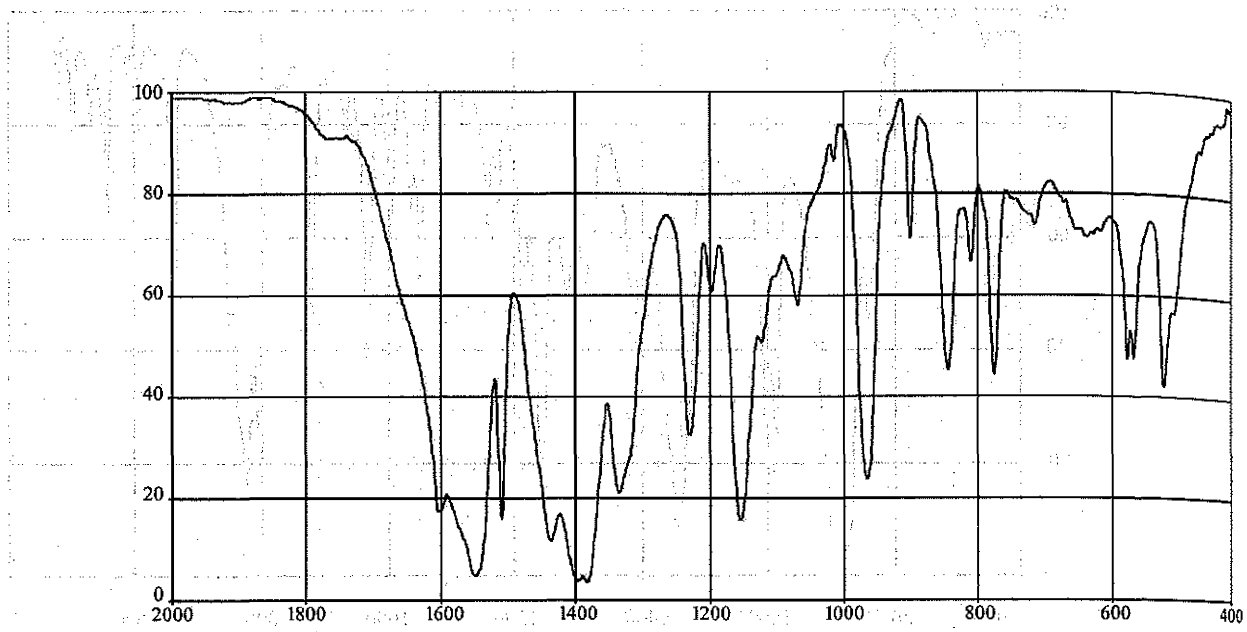
Phase: KBr disc



Rosuvastatin Calcium

FTIR

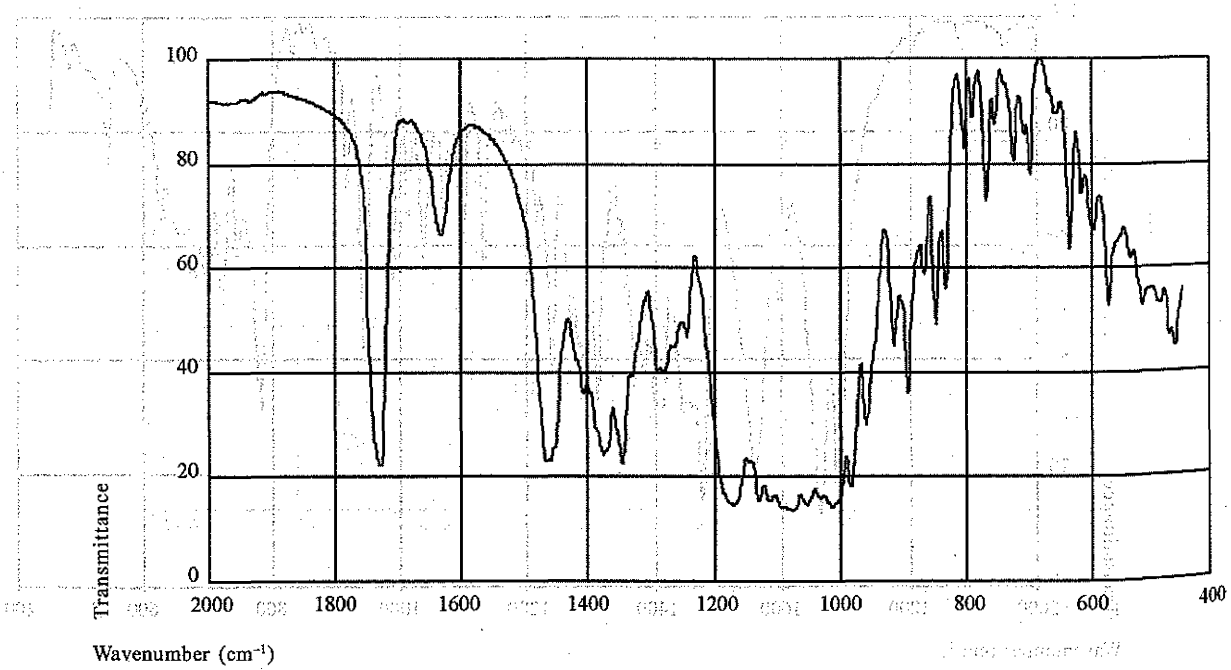
Phase: KBr disc



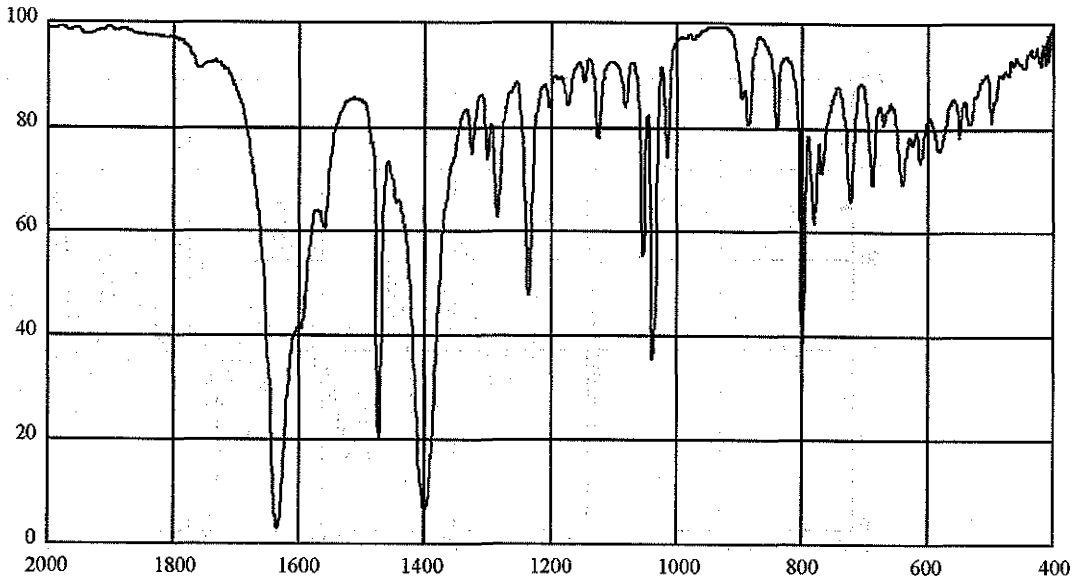
Roxithromycin

FTIR

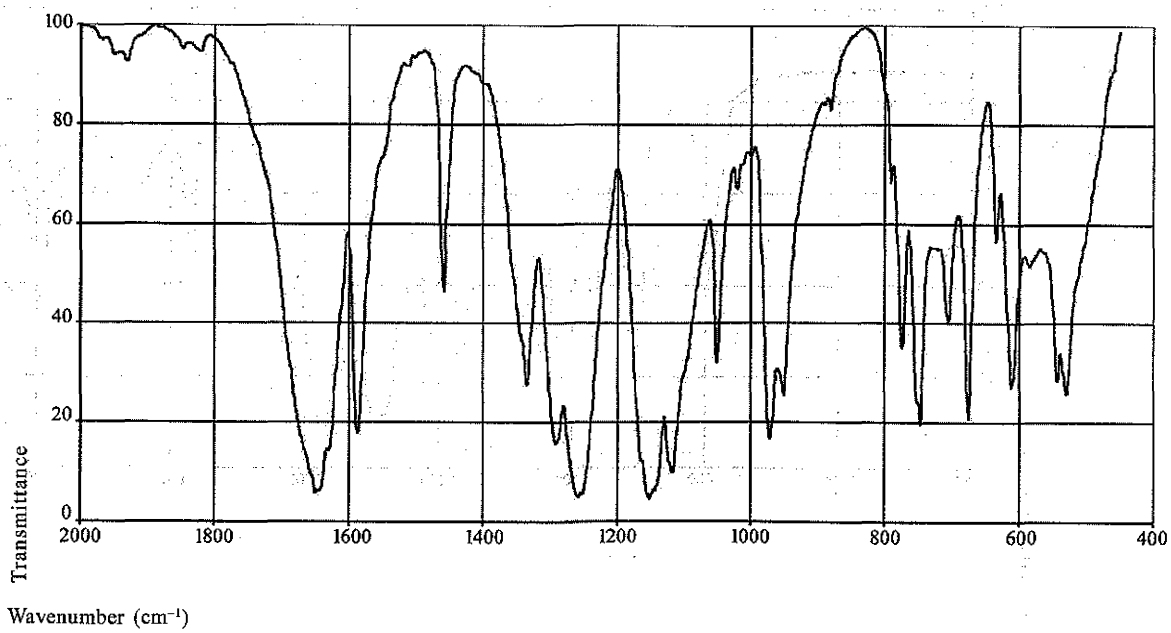
Phase: KBr disc



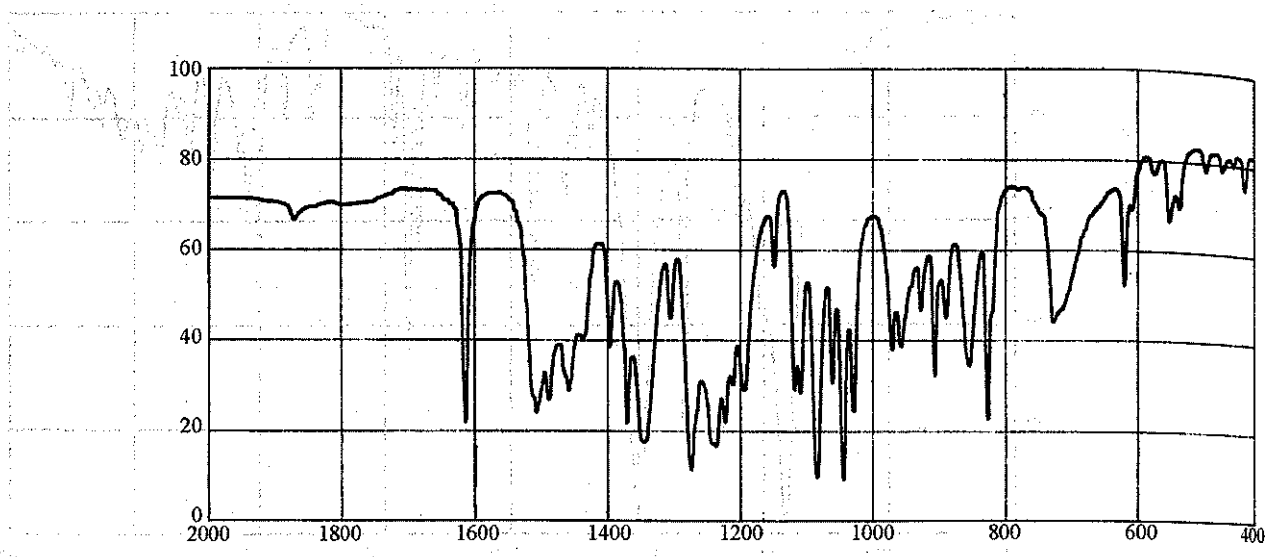
Rufinamide *FTIR* Phase: KBr disc



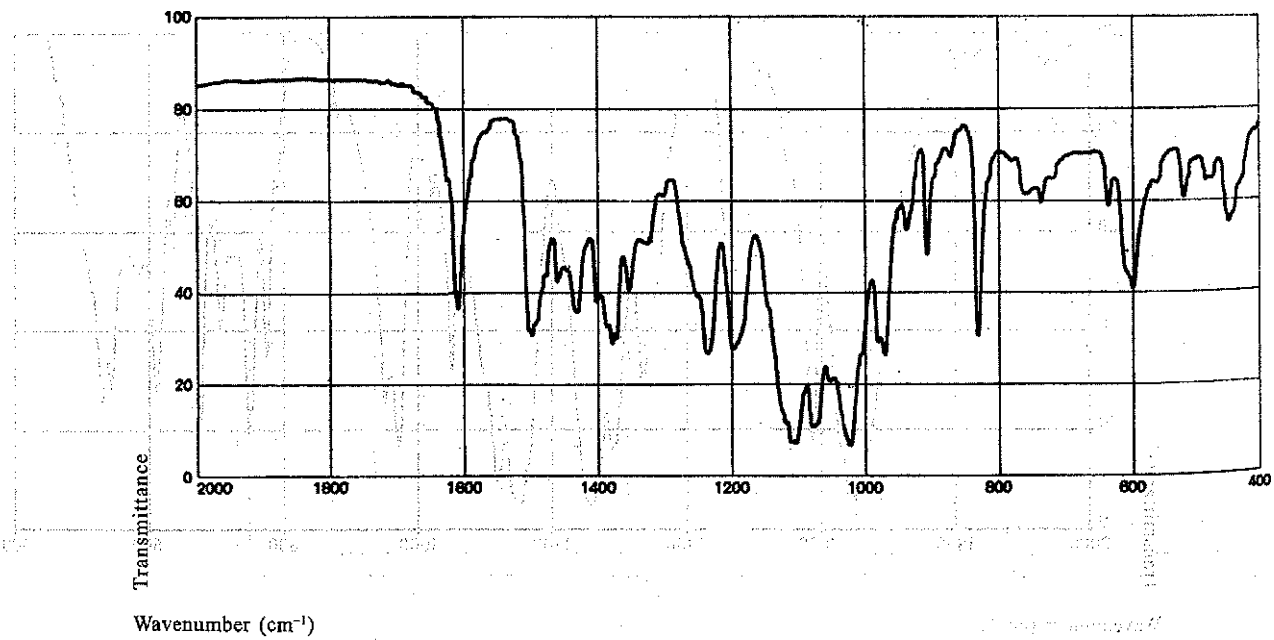
Saccharin Sodium *FTIR* Phase: KBr disc



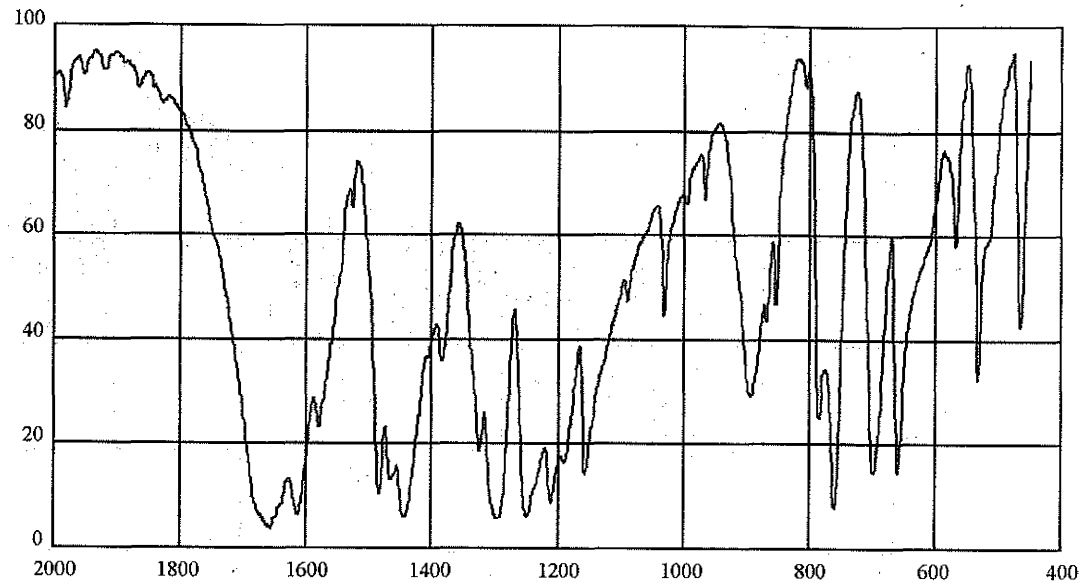
Salbutamol *Dispersive IR* Phase: KBr disc



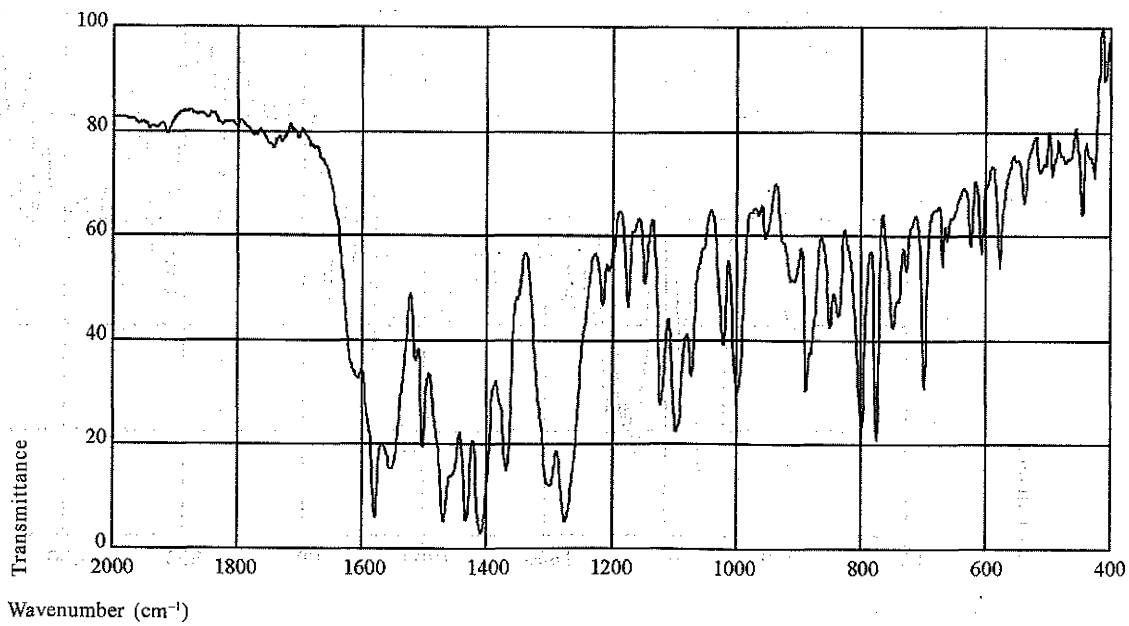
Salbutamol Sulphate *Dispersive IR* Phase: KBr disc



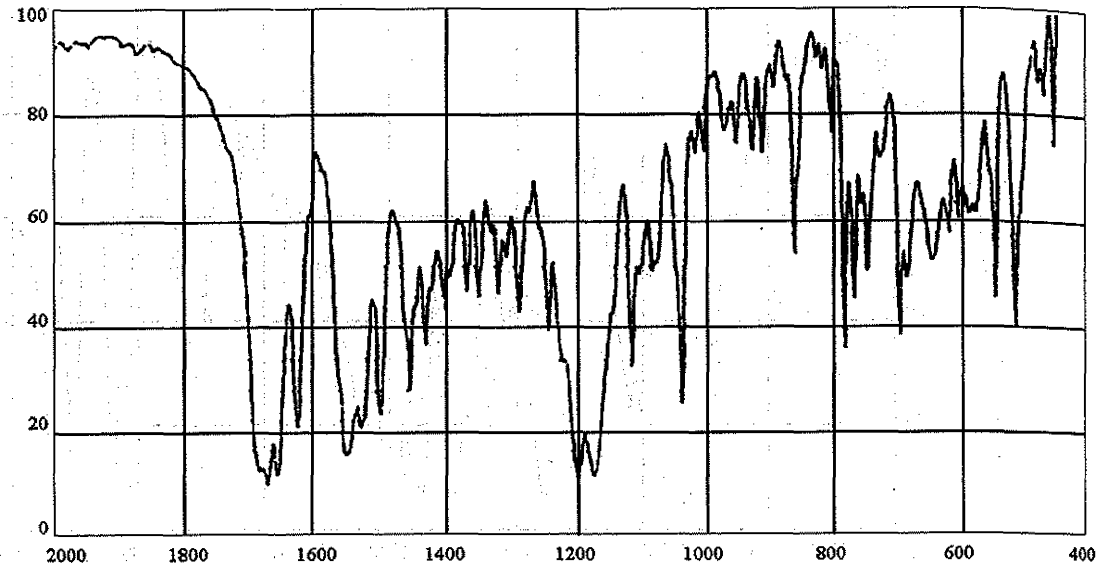
Salicylic Acid *FTIR* Phase: KBr disc



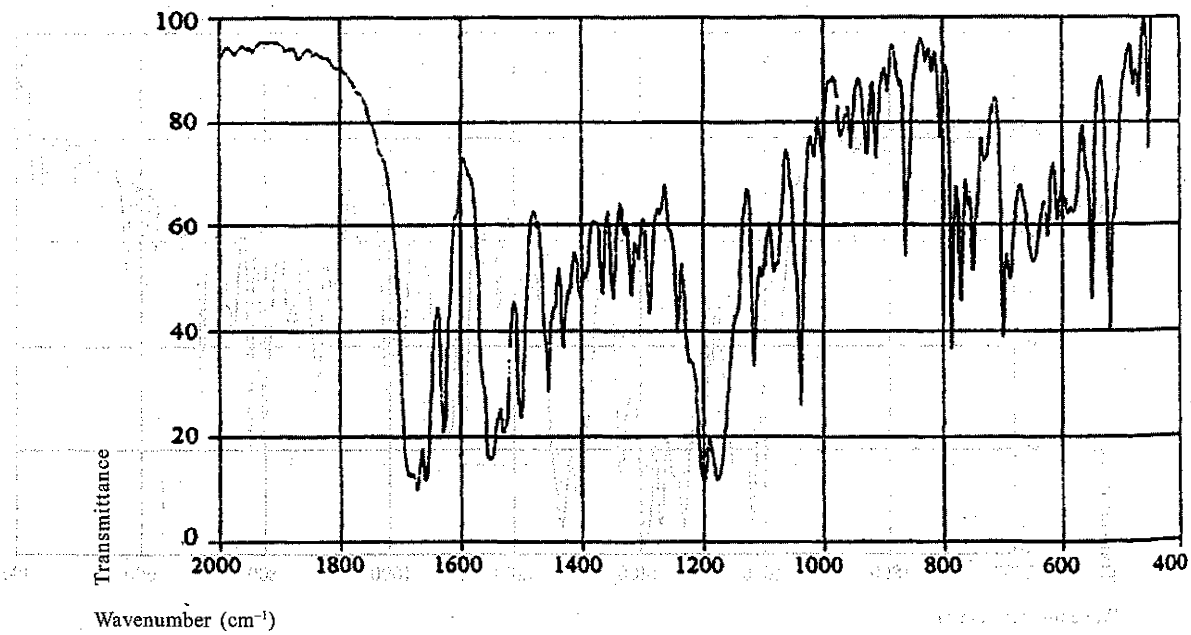
Salmeterol Xinafoate *FTIR* Phase: KBr disc



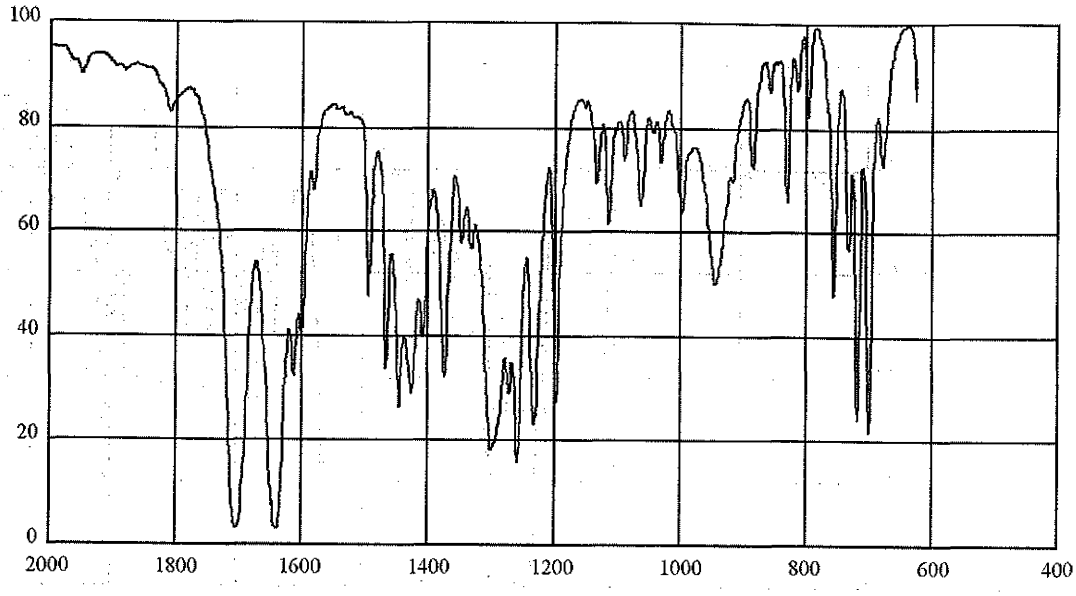
Saquinavir *FTIR* Phase: KBr disc



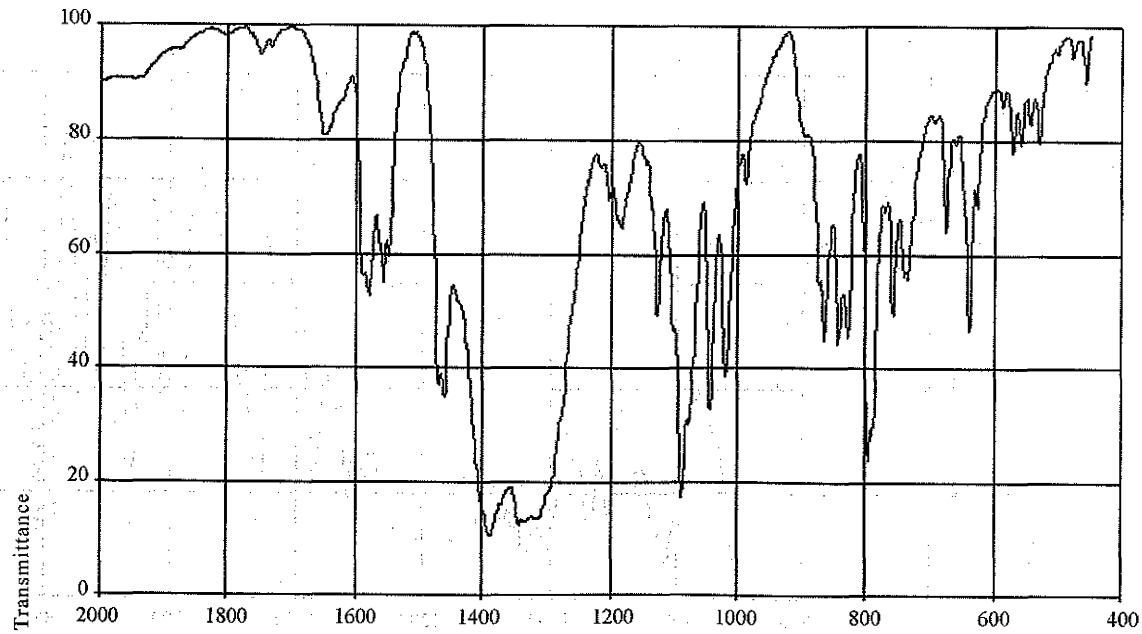
Saquinavir Mesylate *FTIR* Phase: KBr disc



Seratrodast *FTIR* Phase: KBr disc



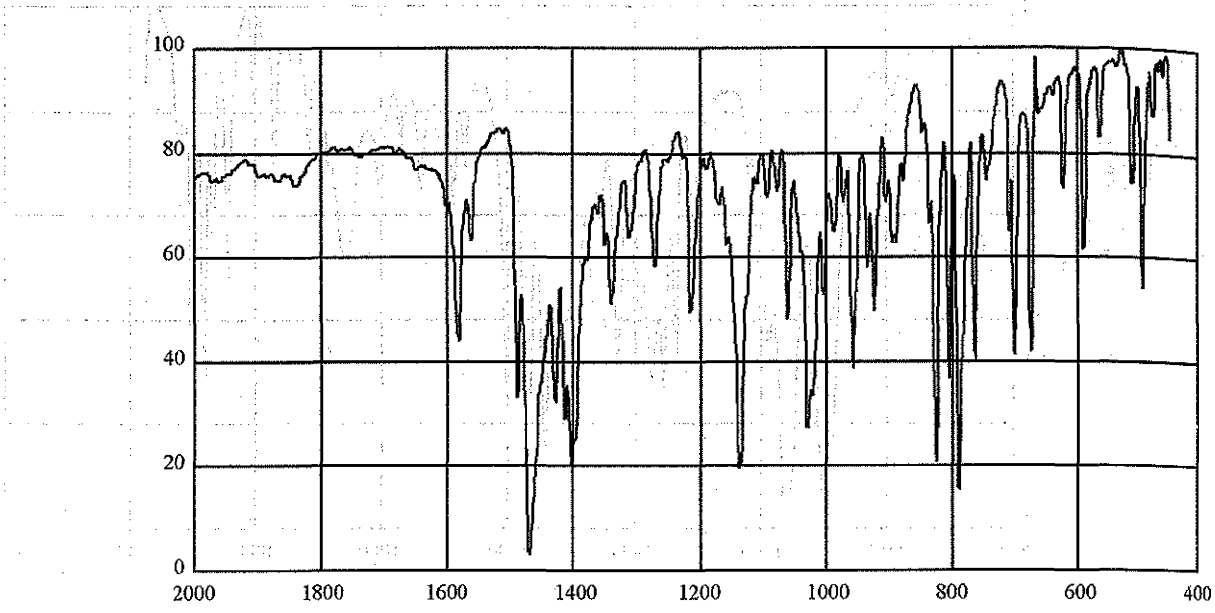
Sertaconazole Nitrate *FTIR* Phase: KBr disc



Sertraline Hydrochloride

FTIR

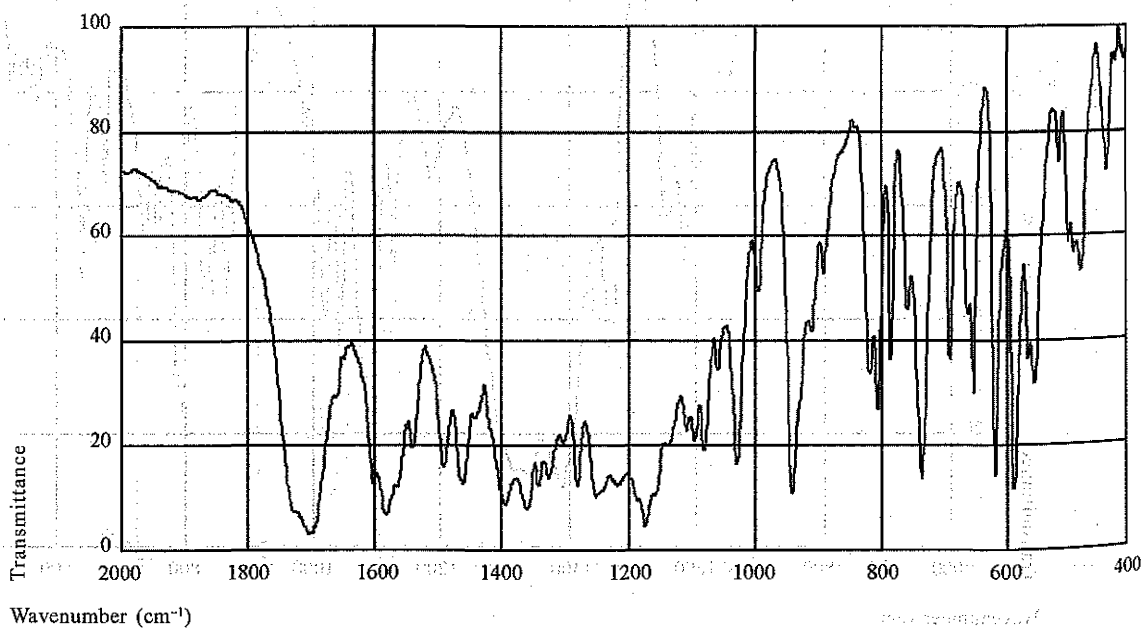
Phase: KBr disc



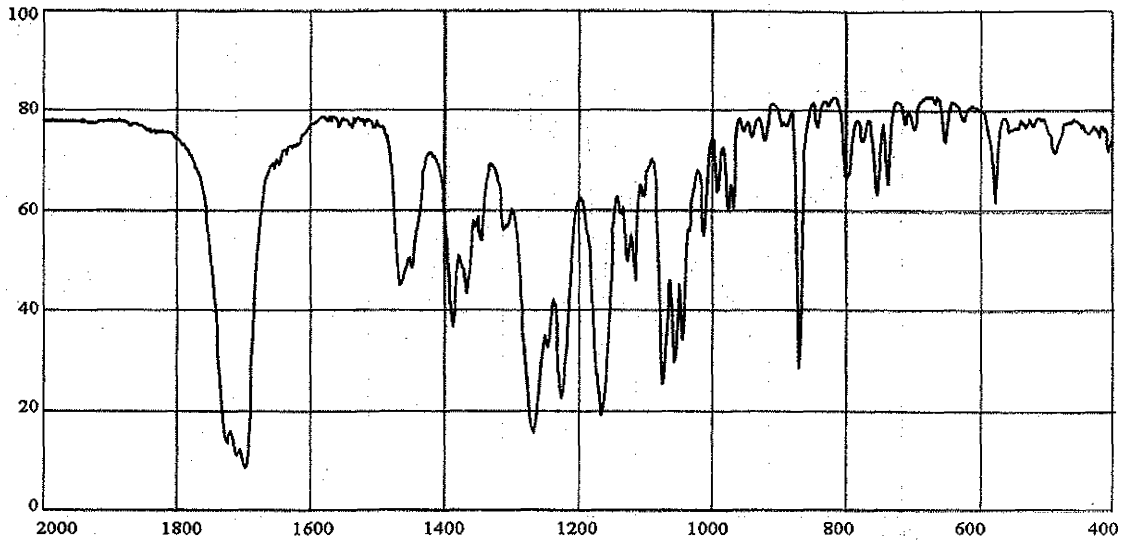
Sildenafil Citrate

FTIR

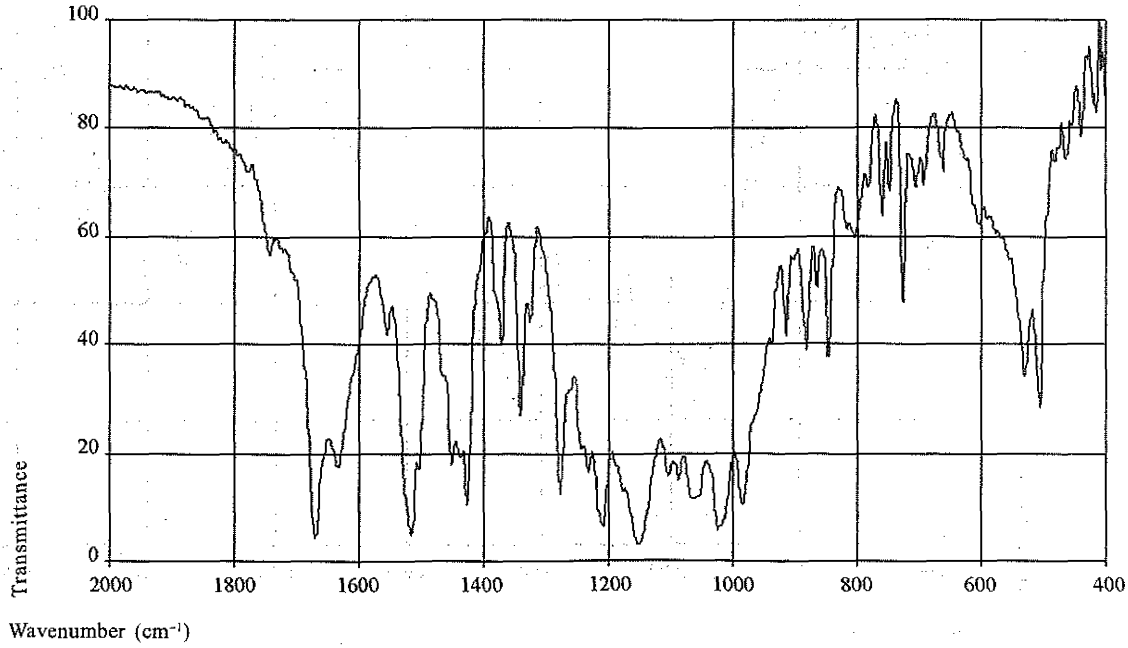
Phase: KBr disc



Simvastatin *FTIR* Phase: KBr disc



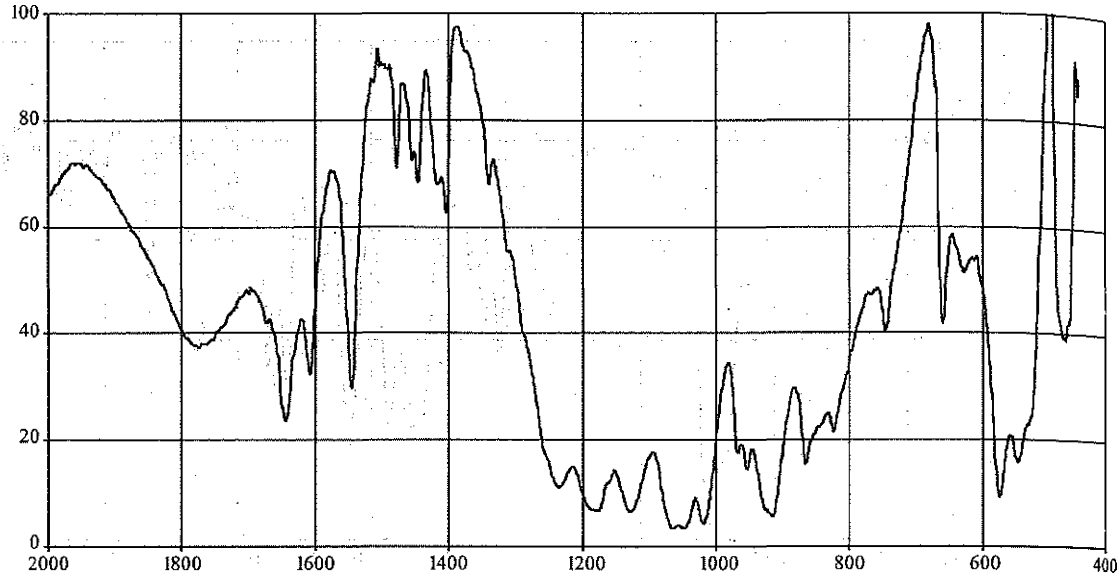
Sitagliptin Phosphate *FTIR* Phase: KBr disc



Sodium Alendronate Trihydrate

FTIR

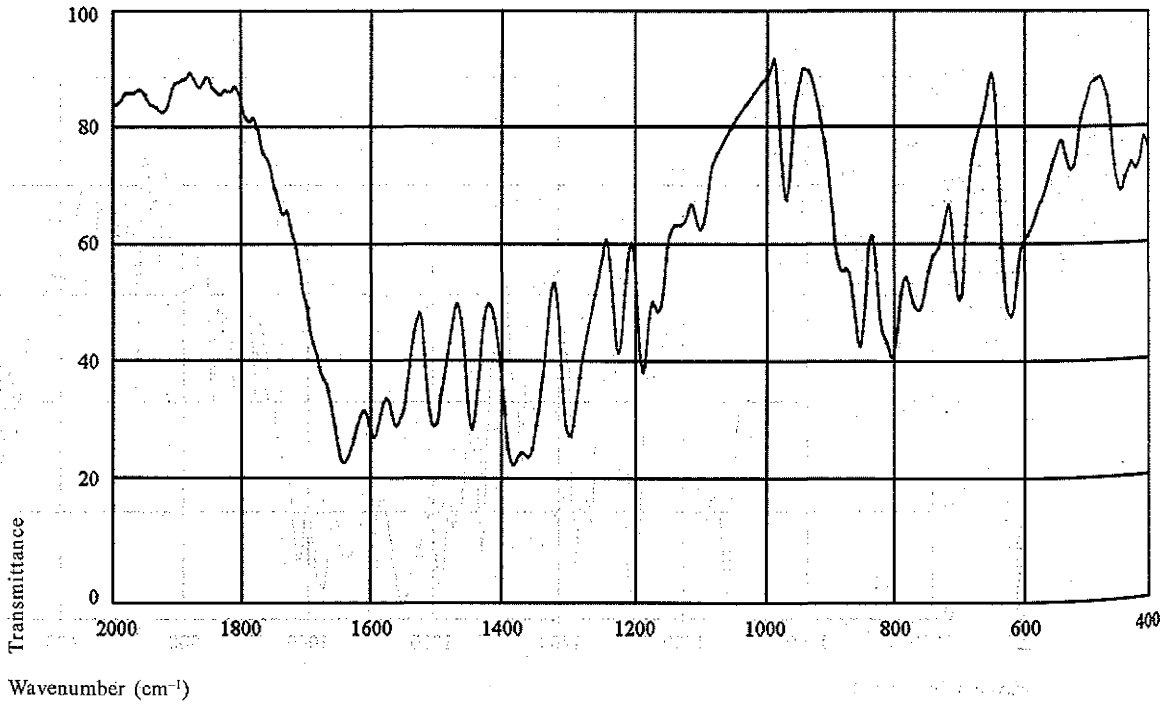
Phase: KBr disc



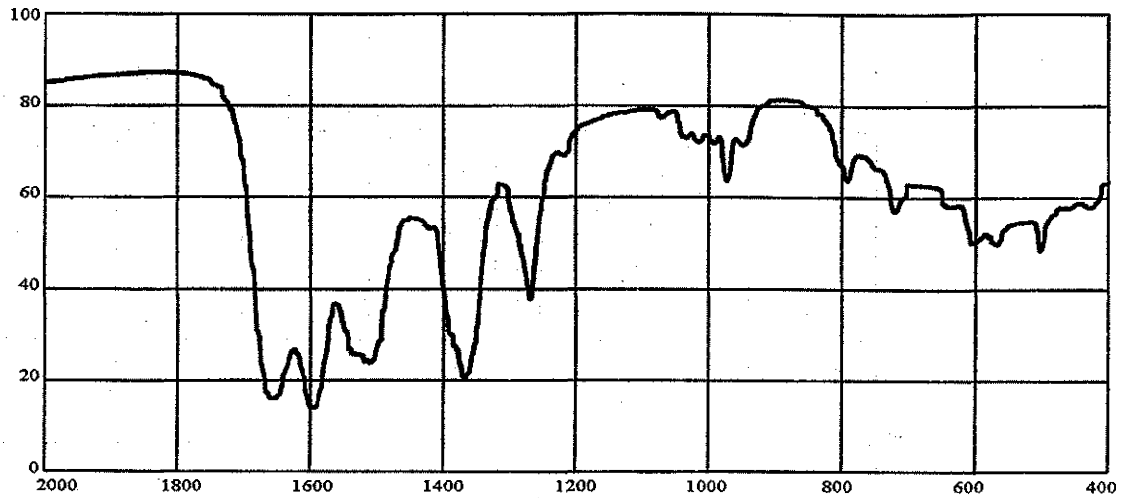
Sodium Aminosalicylate

FTIR

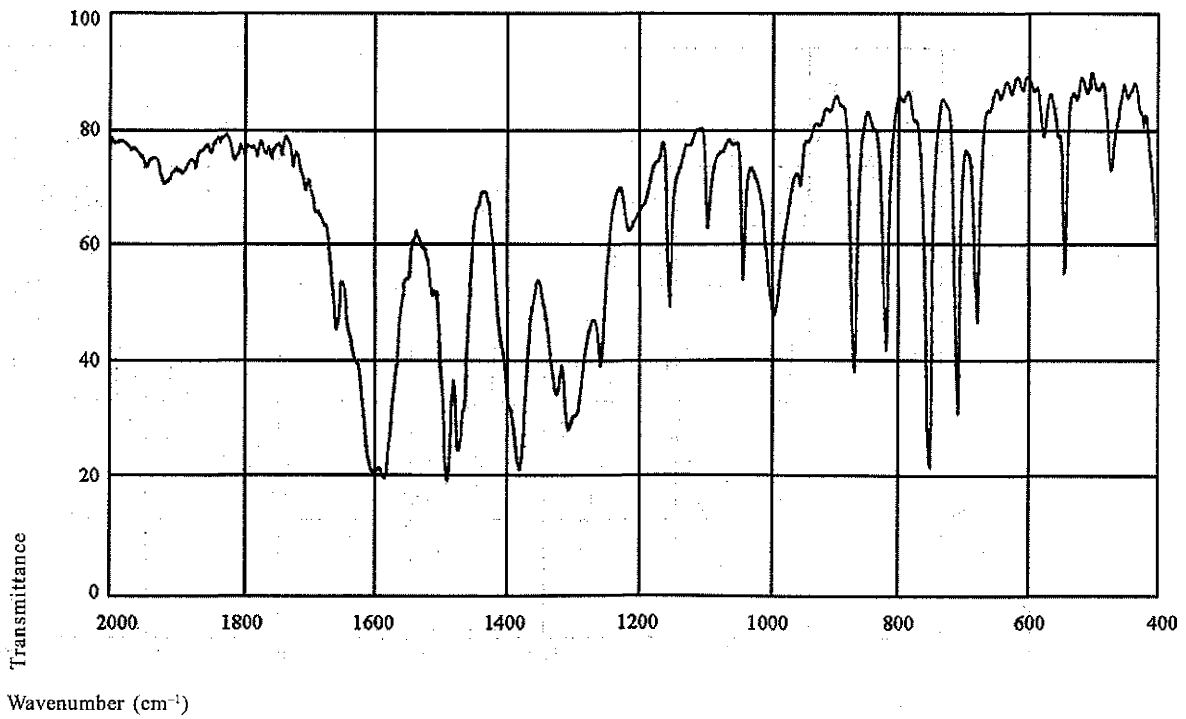
Phase: KBr disc



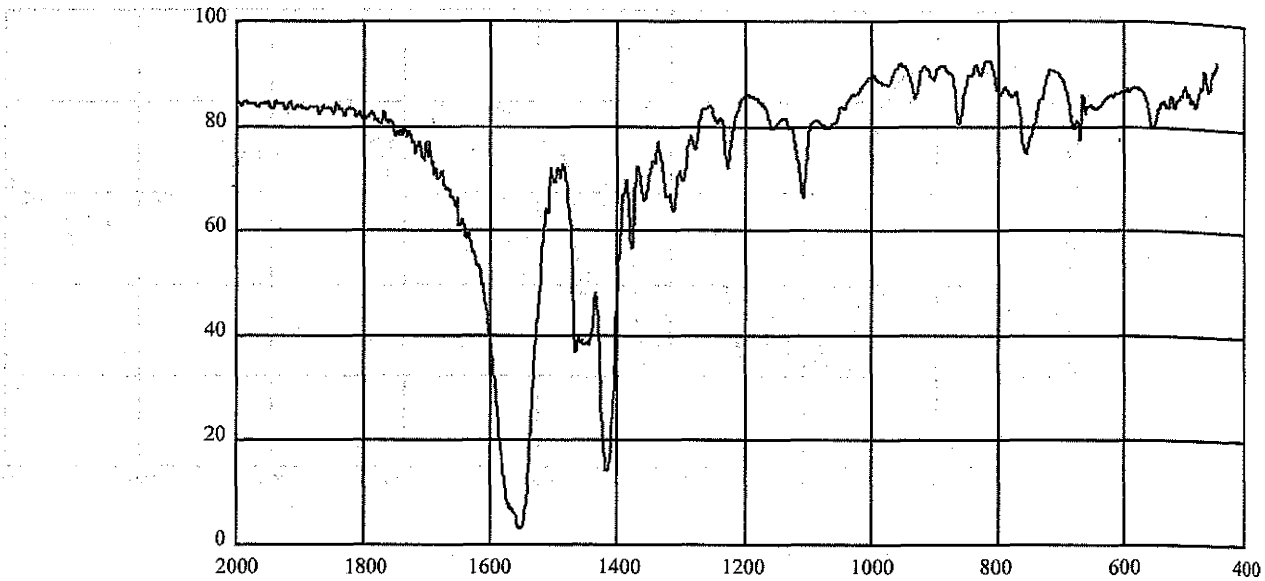
Sodium Diatrizoate *Dispersive IR* Phase: KBr disc



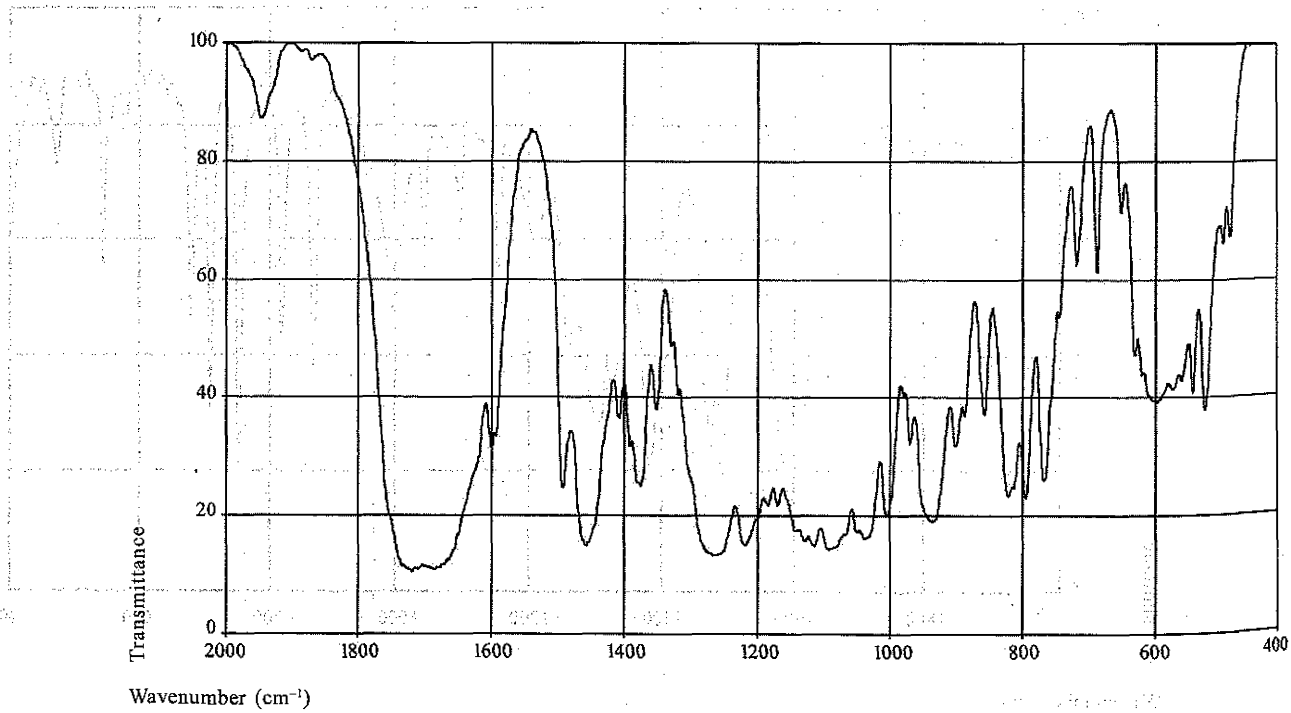
Sodium Salicylate *FTIR* Phase: KBr disc



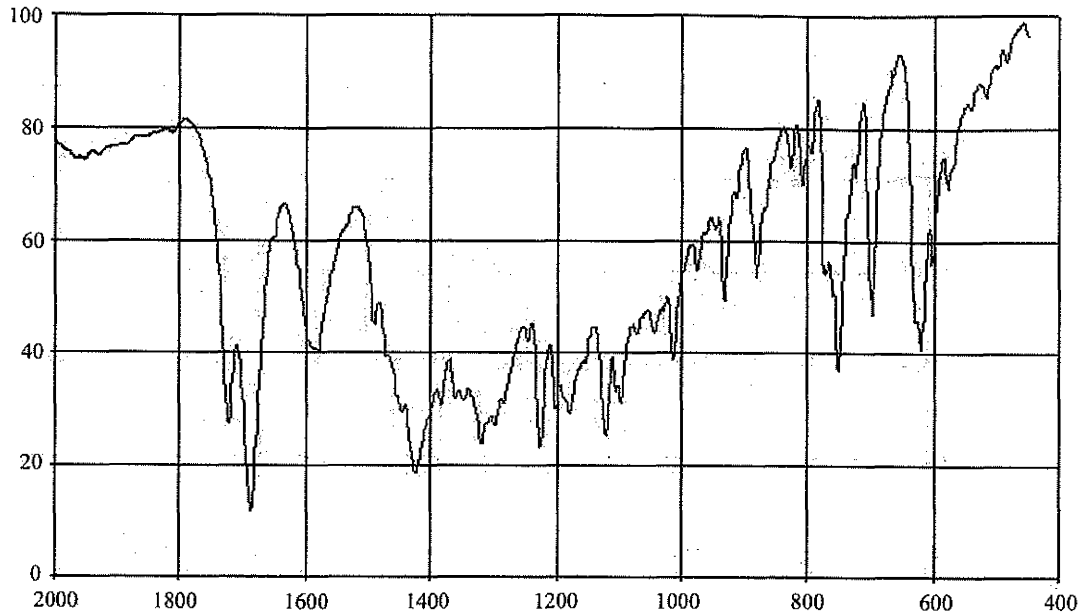
Sodium Valproate *FTIR* Phase: KBr disc



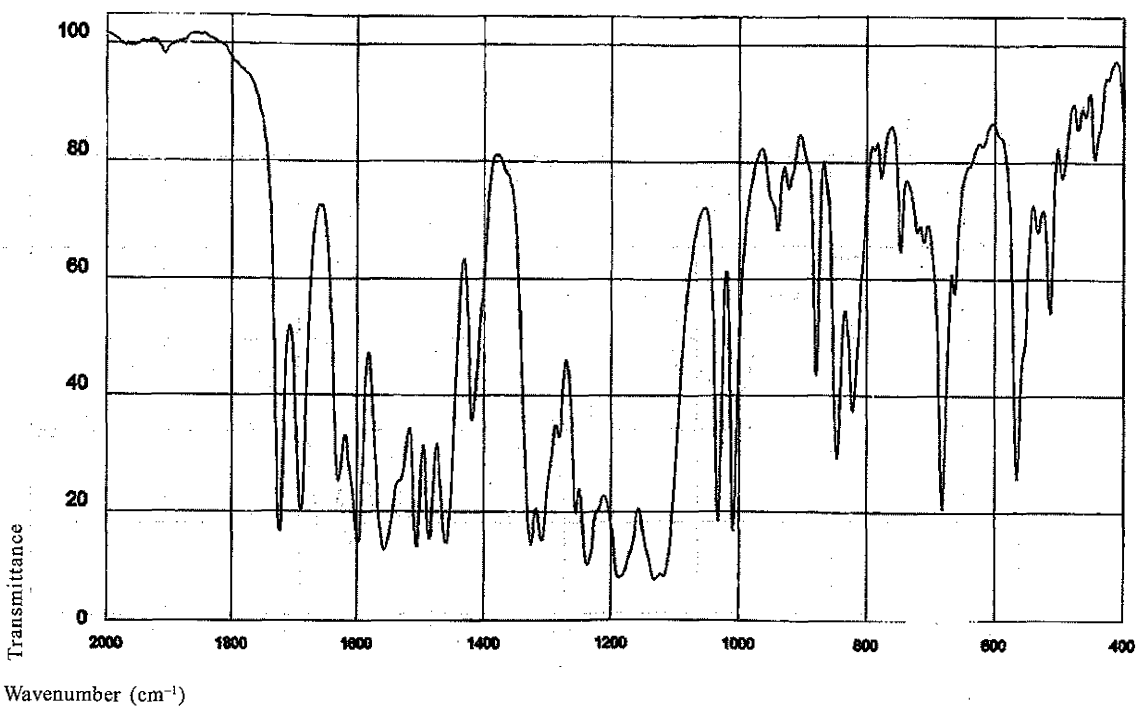
Sofosbuvir *FTIR* Phase: KBr disc



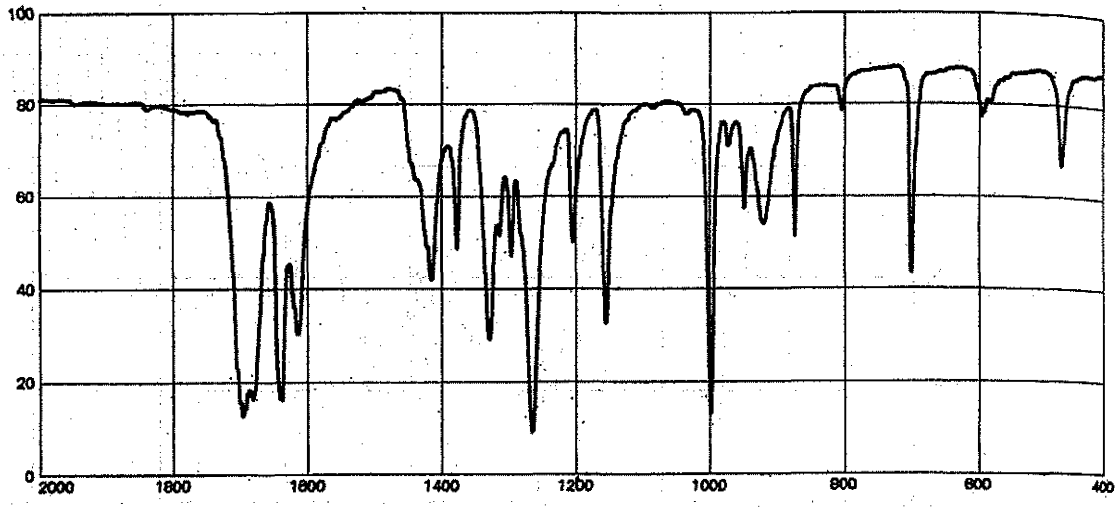
Solifenacin Succinate *FTIR* Phase: KBr disc



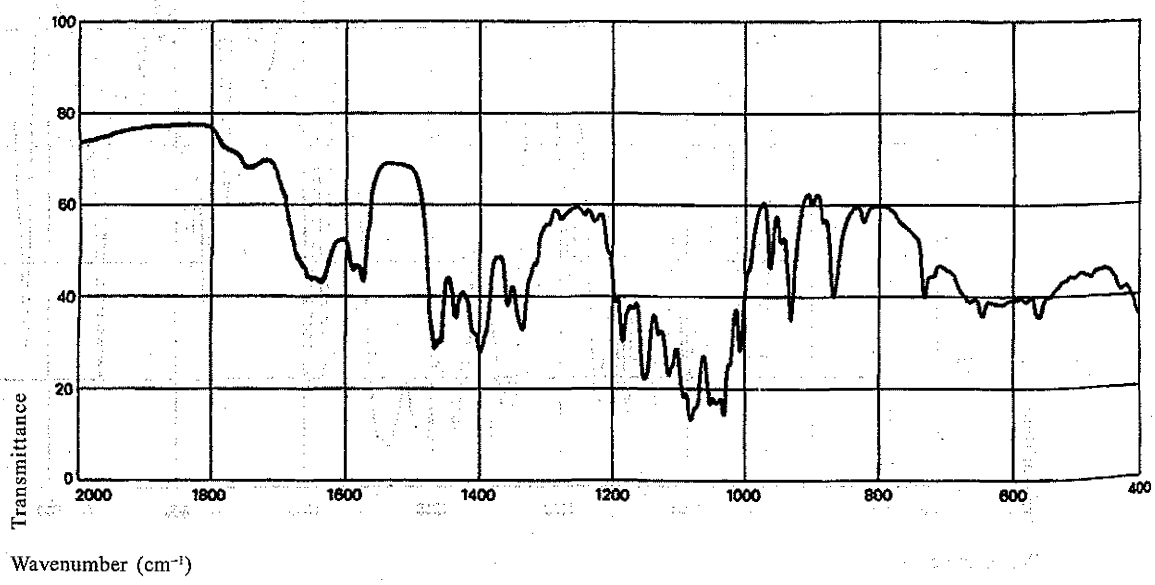
Sorafenib Tosylate *FTIR* Phase: KBr disc



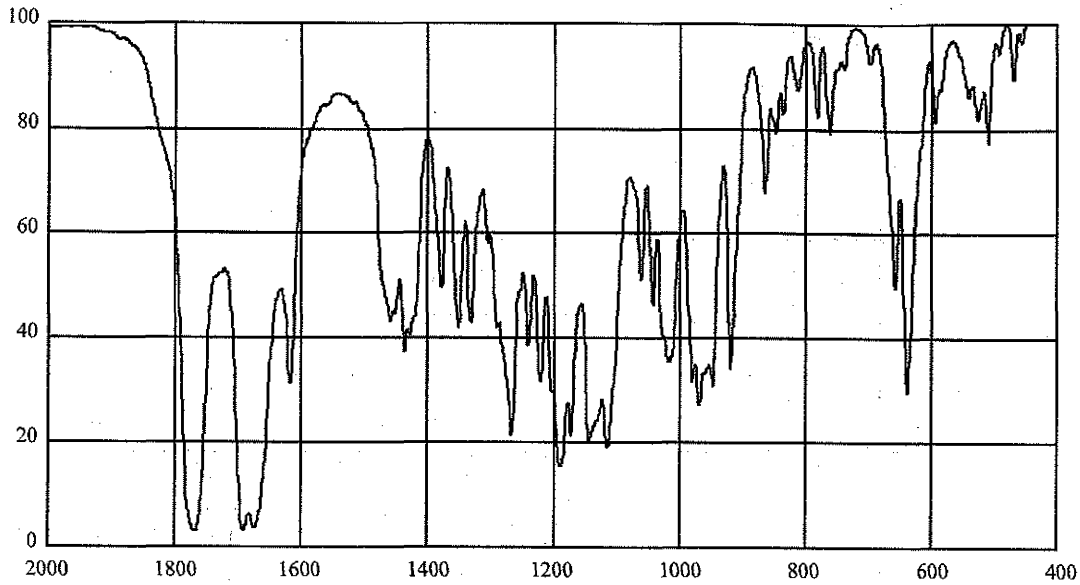
Sorbic Acid *Dispersive IR* Phase: KBr disc



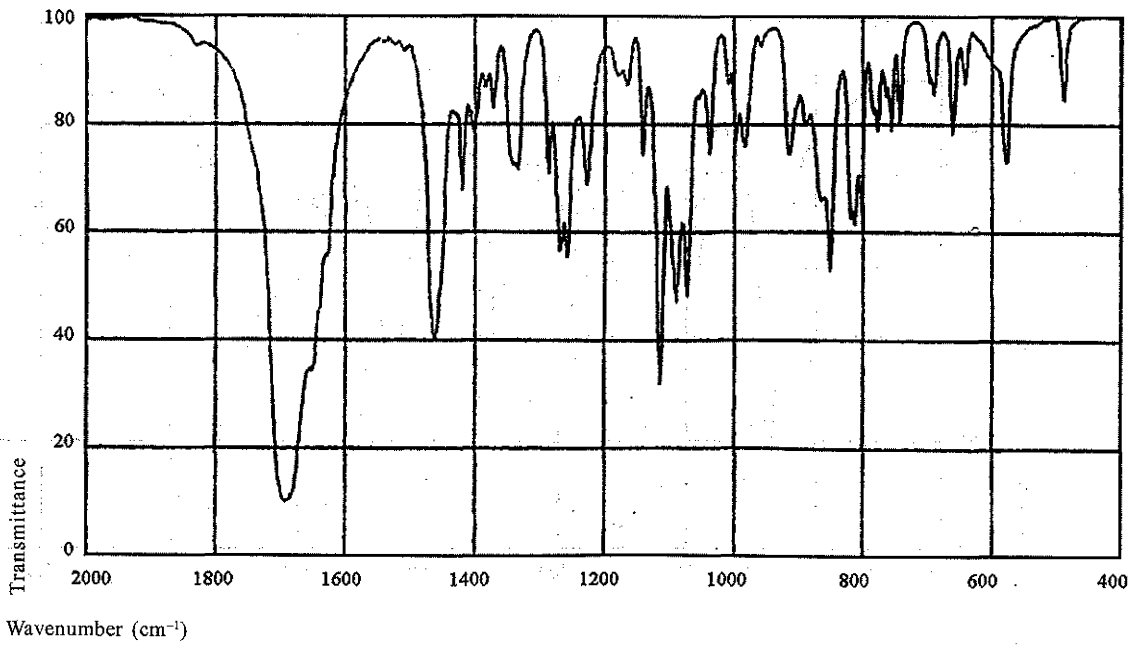
Spectinomycin Hydrochloride *Dispersive IR* Phase: KCl disc



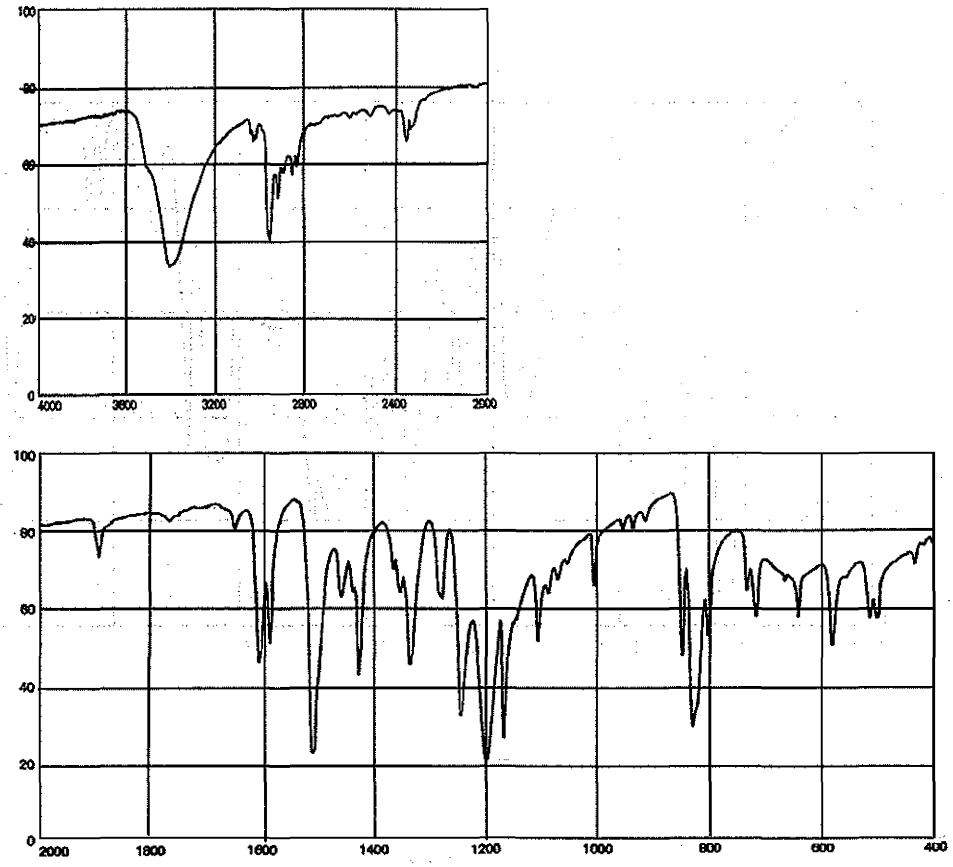
Spironolactone *FTIR* Phase: KBr disc



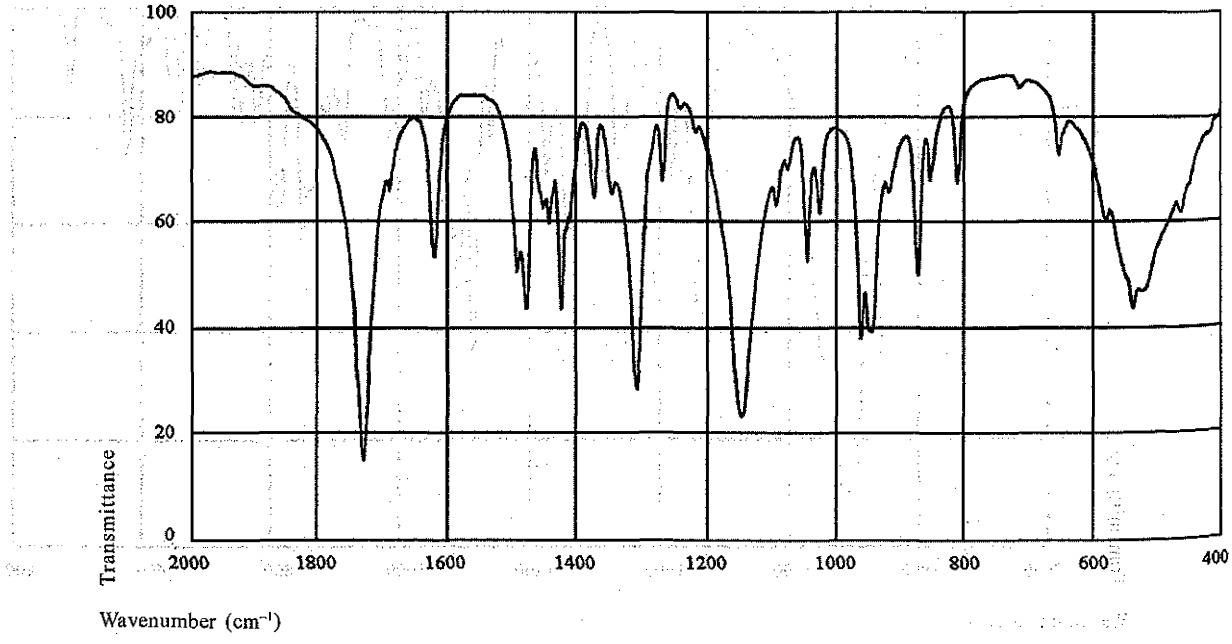
Stavudine *FTIR* Phase: KBr disc



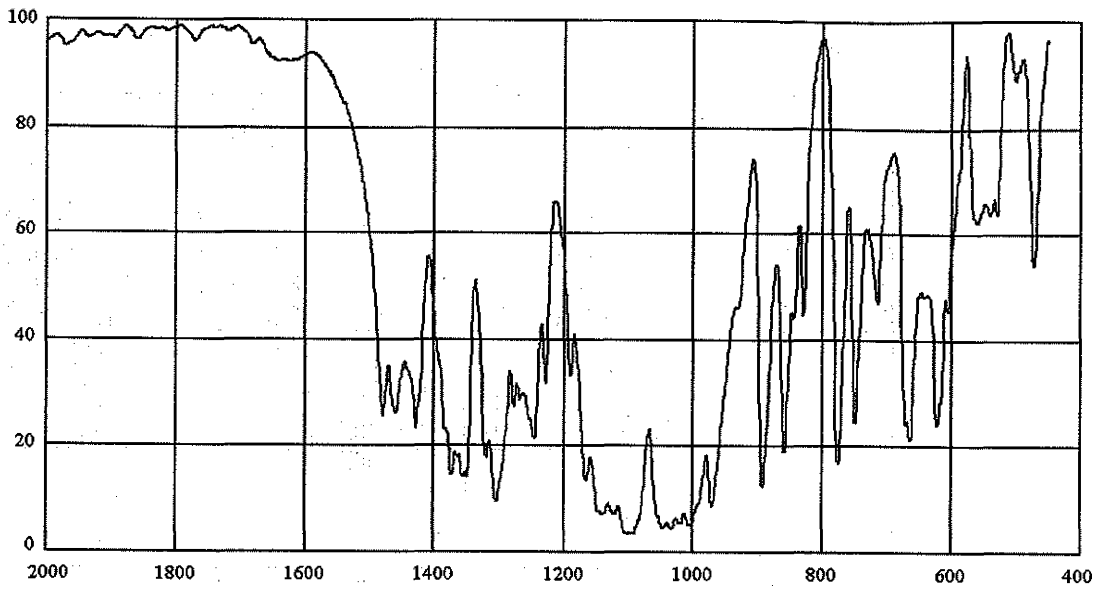
Stilboestrol *FTIR* Phase: KBr disc



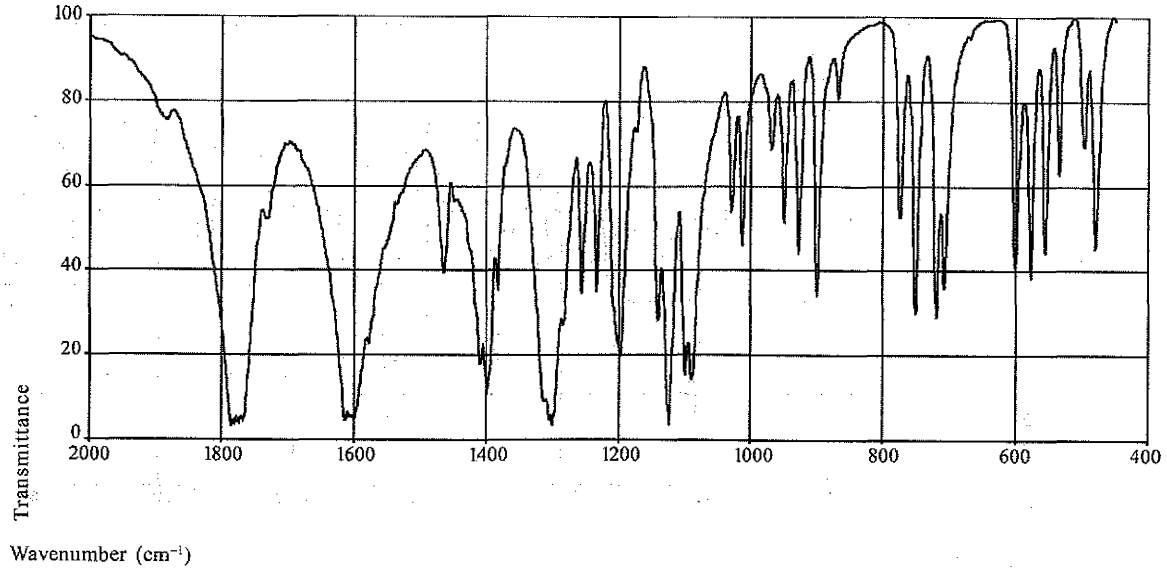
Succinylcholine Chloride *FTIR* Phase: KBr disc



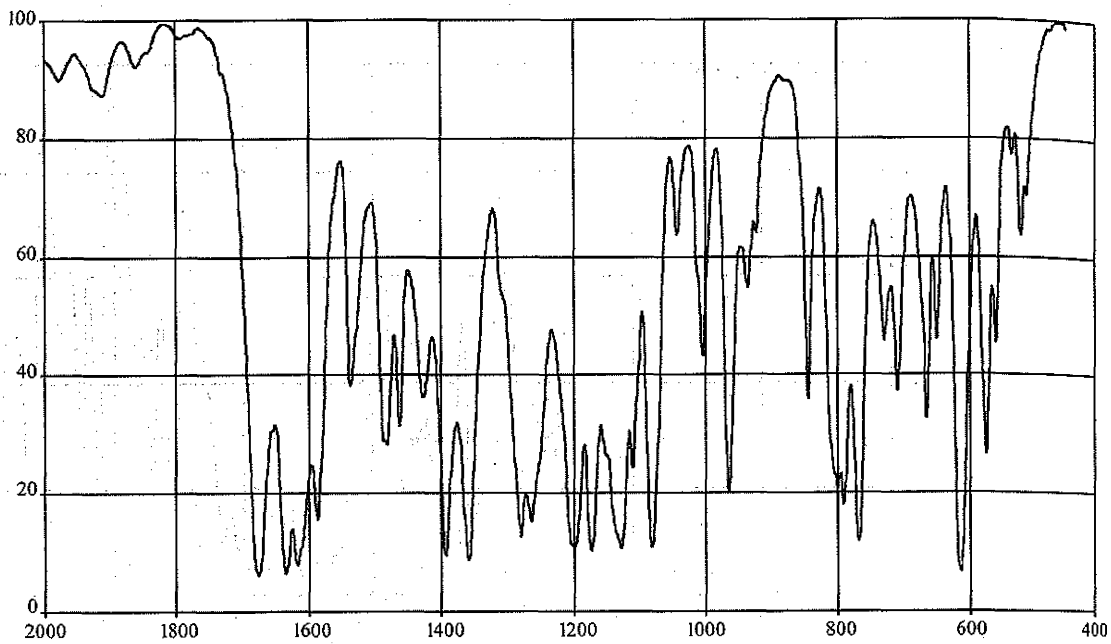
Sucralose *FTIR* Phase: Liquid paraffin mull



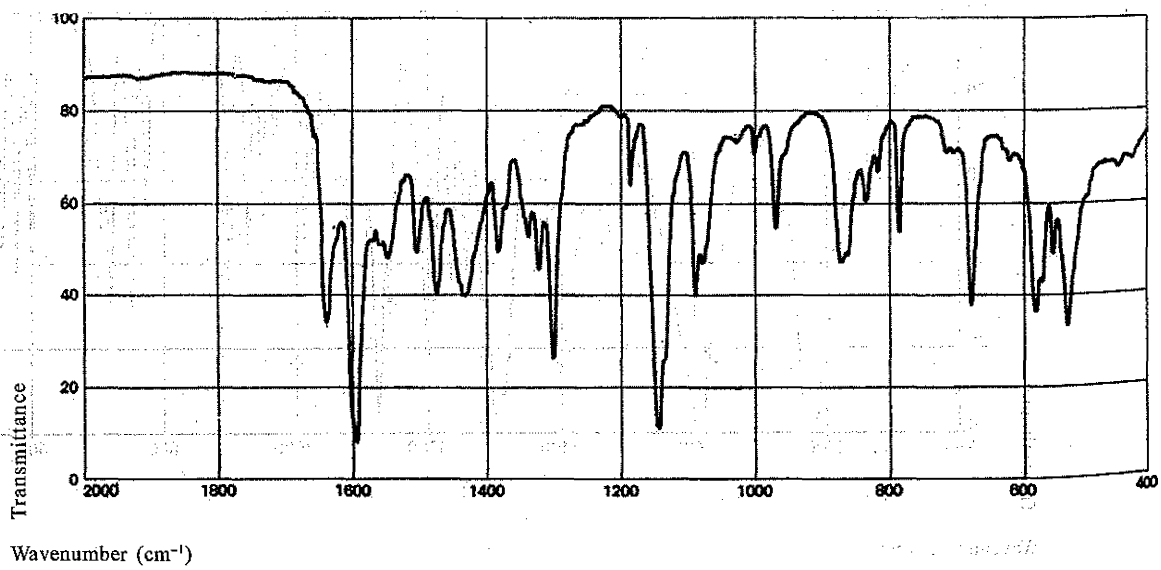
Sulbactam Sodium *FTIR* Phase: KBr disc



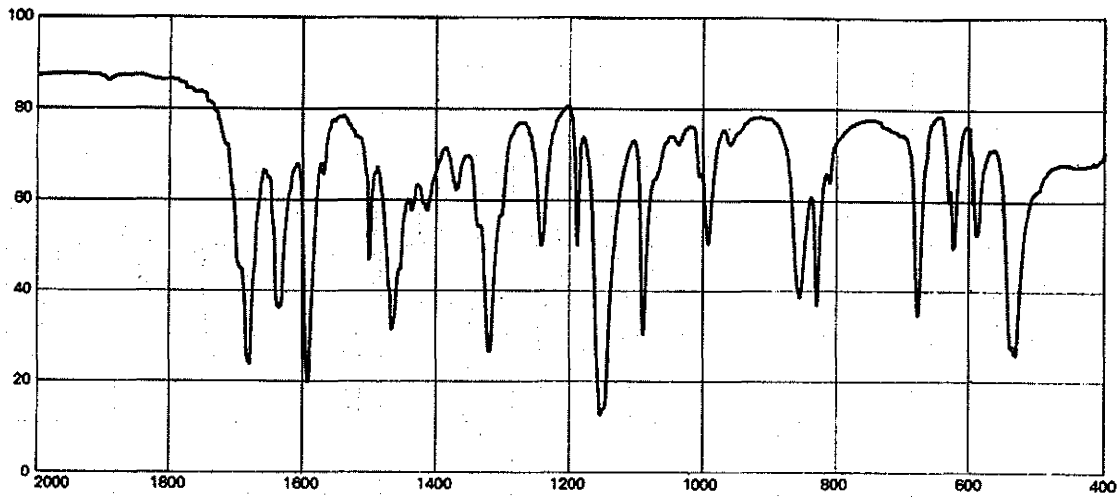
Sulfasalazine *FTIR* Phase: KBr disc



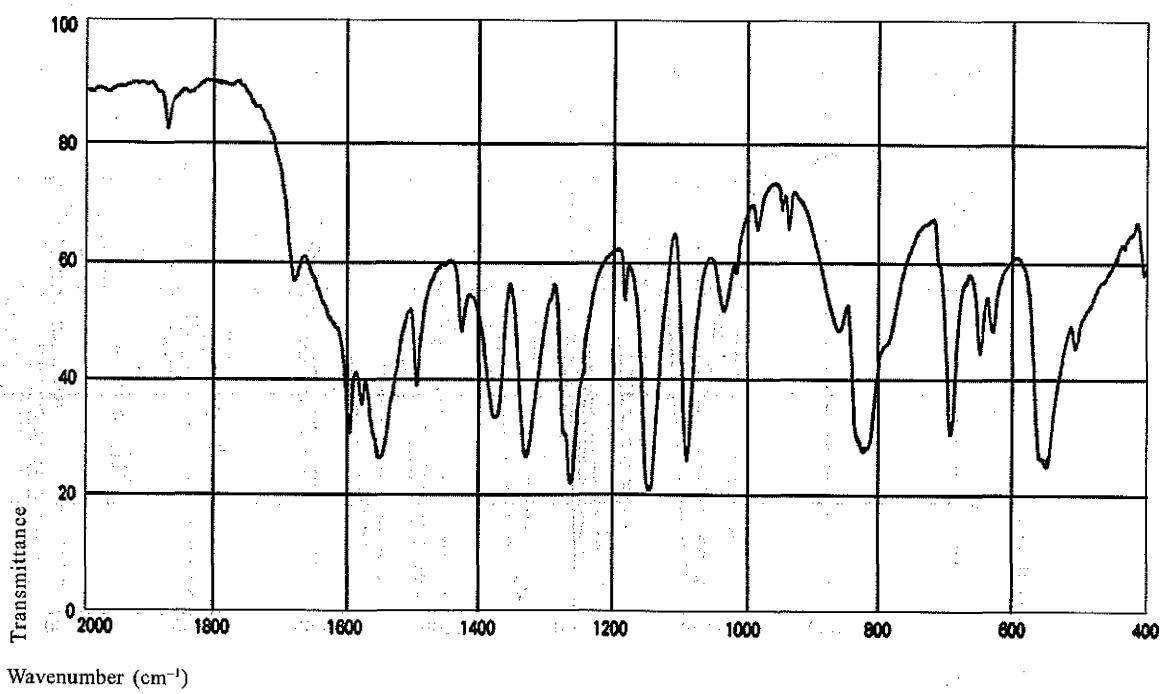
Sulphadimidine *Dispersive IR* Phase: KBr disc



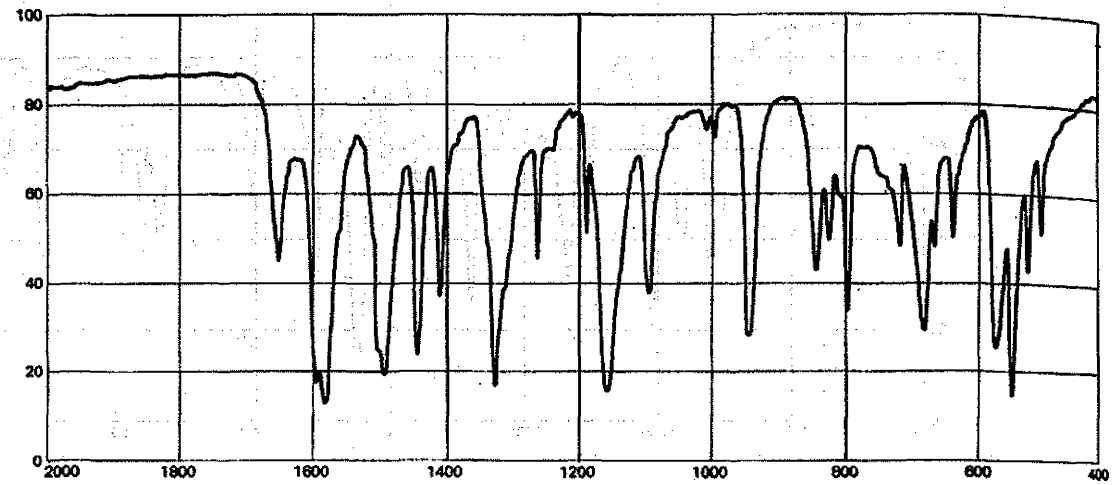
Sulphacetamide *Dispersive IR* Phase: KBr disc



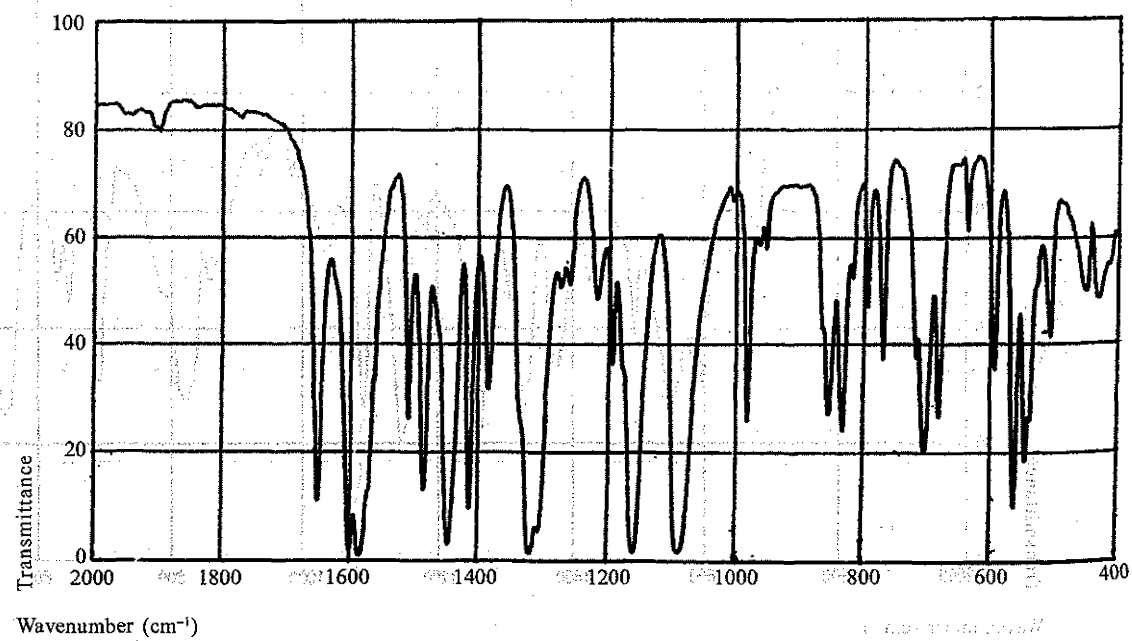
Sulphacetamide Sodium *FTIR* Phase: KBr disc



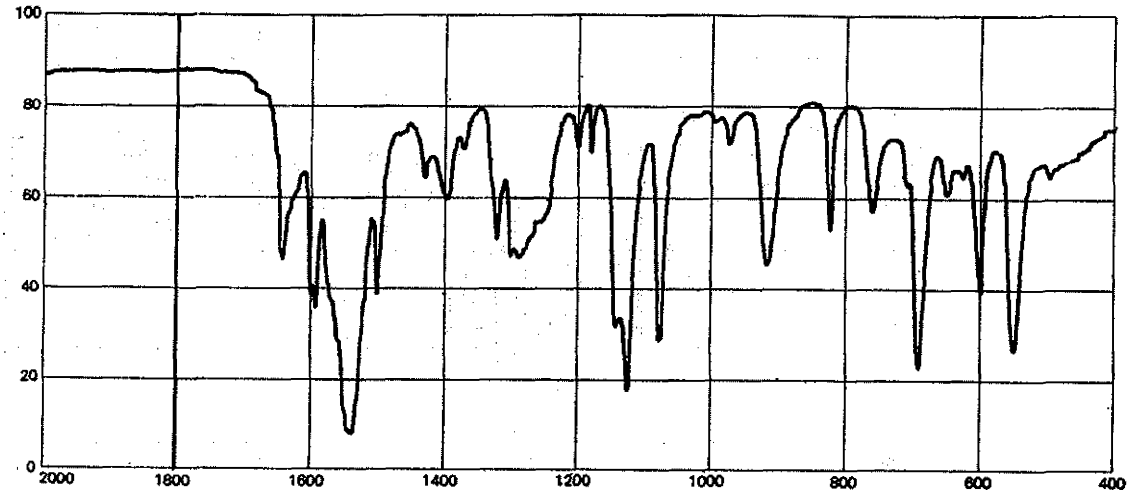
Sulphadiazine *Dispersive IR* Phase: KBr disc



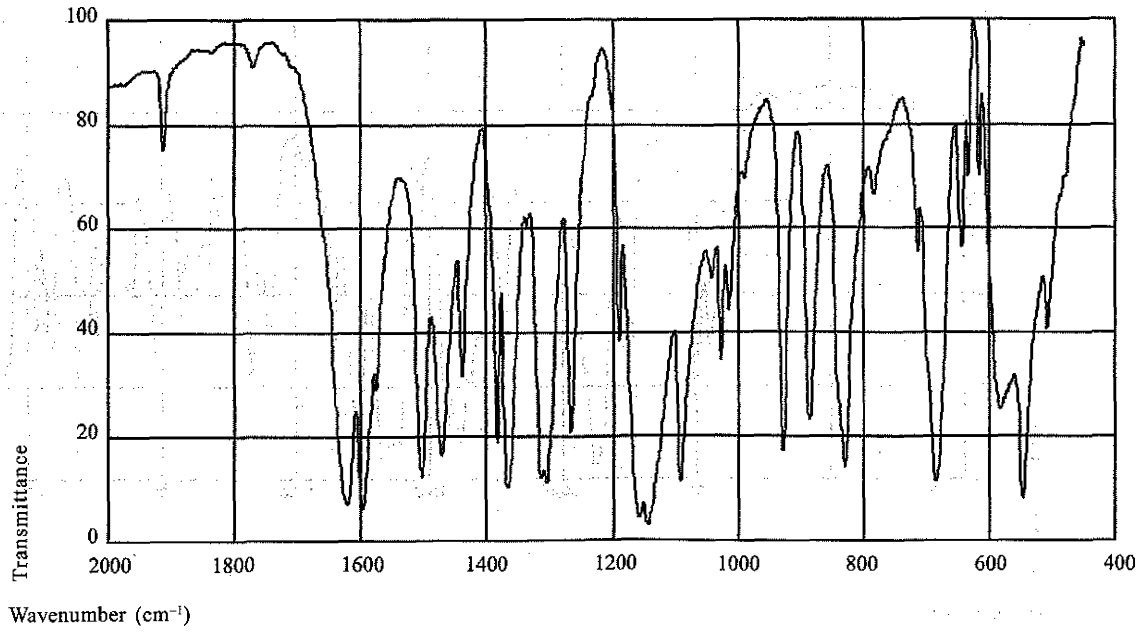
Sulphadoxine *FTIR* Phase: KBr disc



Sulphamethizole *Dispersive IR* Phase: KBr disc



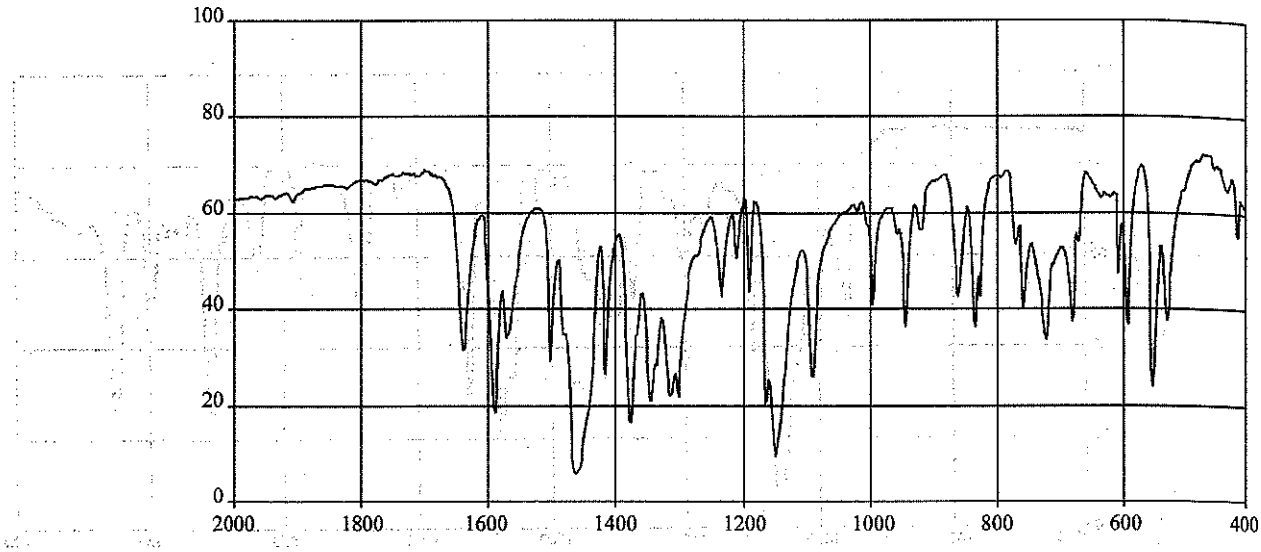
Sulphamethoxazole *FTIR* Phase: KBr disc



Sulphaquinoxaline

FTIR

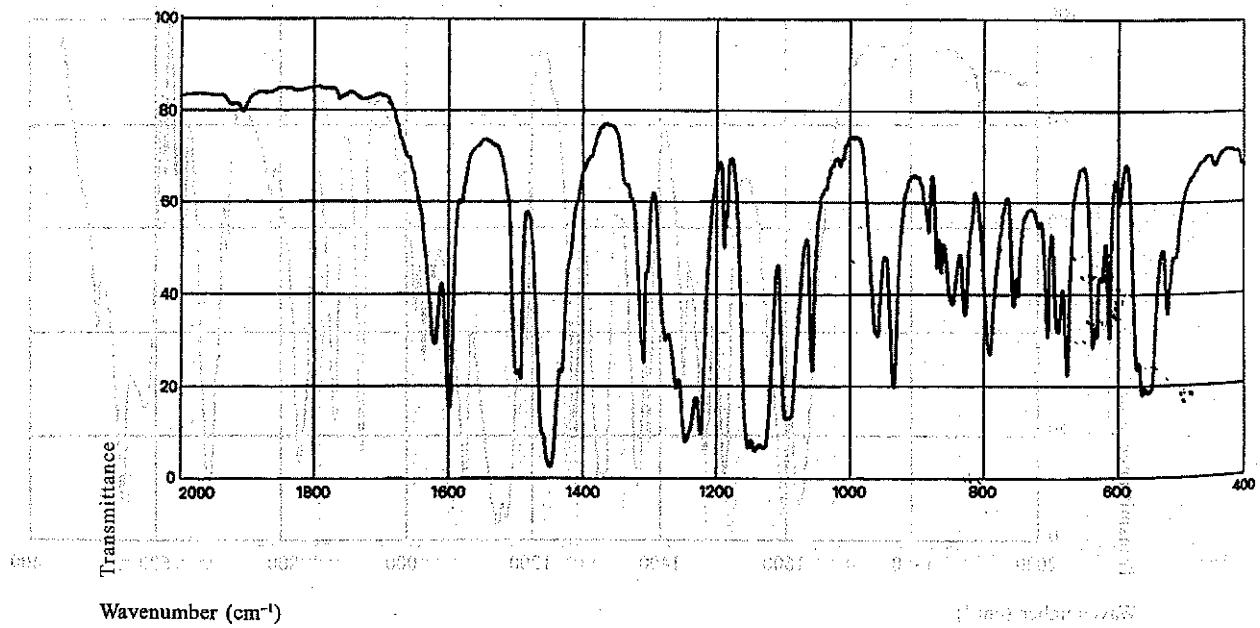
Phase: Liquid paraffin mull



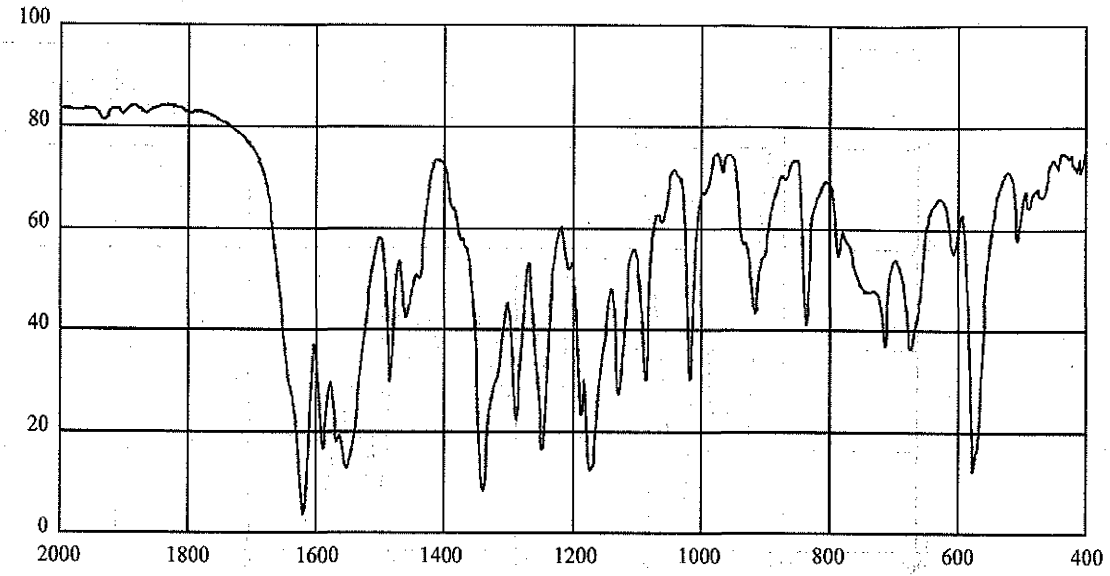
Sulphathiazole Sodium

Dispersive IR

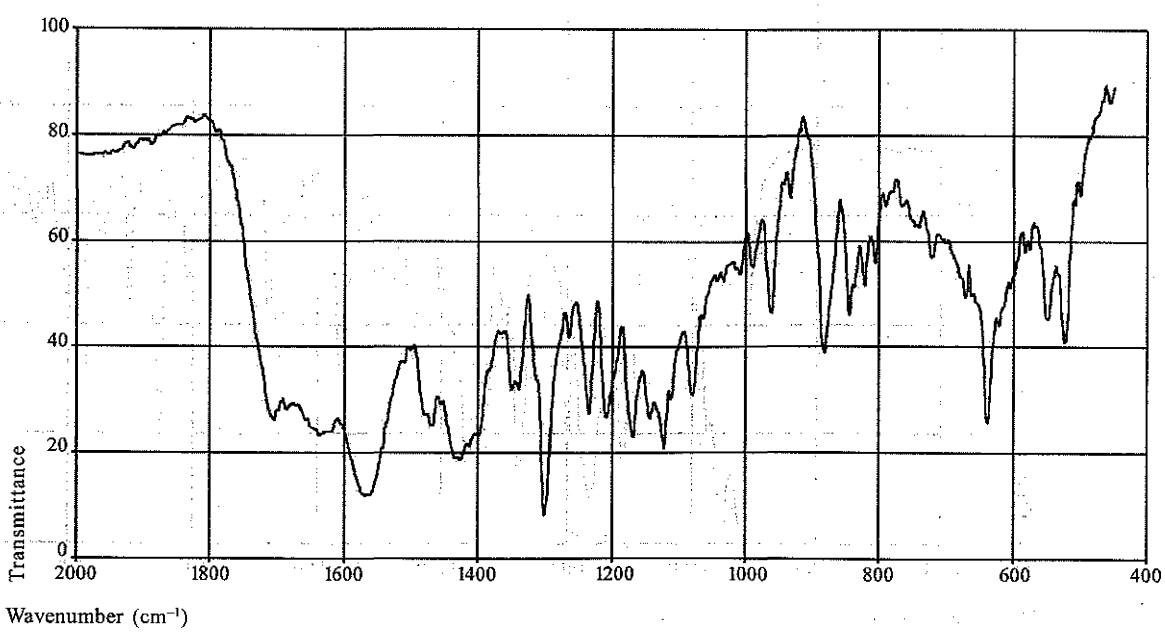
Phase: KBr disc



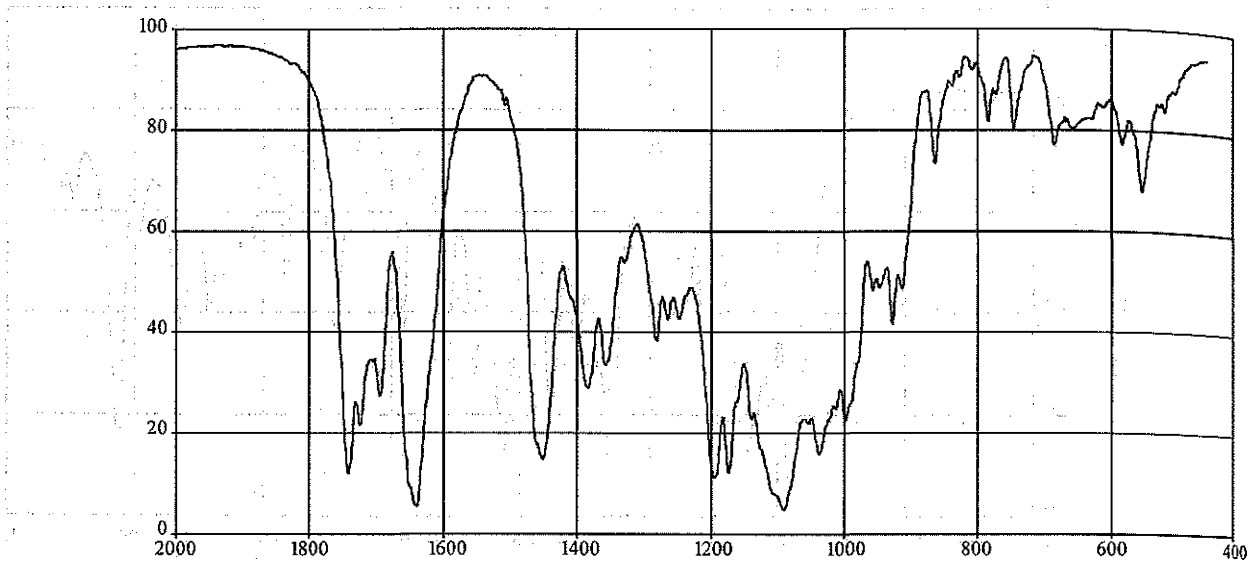
Sulpiride *FTIR* Phase: KBr disc



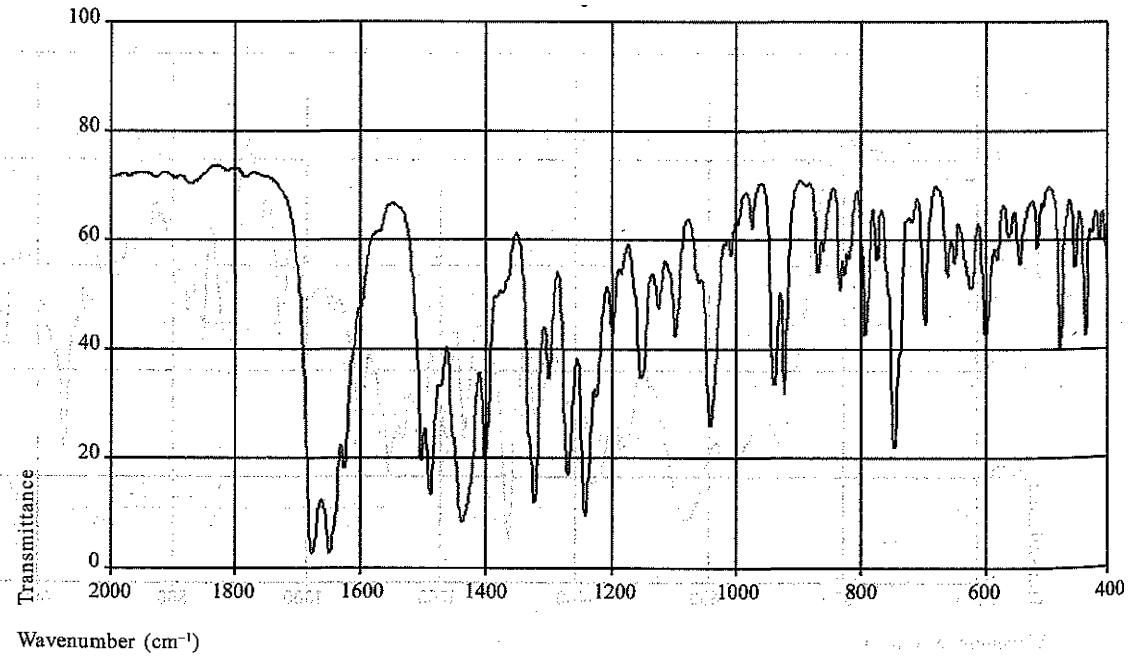
Sumatriptan Succinate *FTIR* Phase: KBr disc



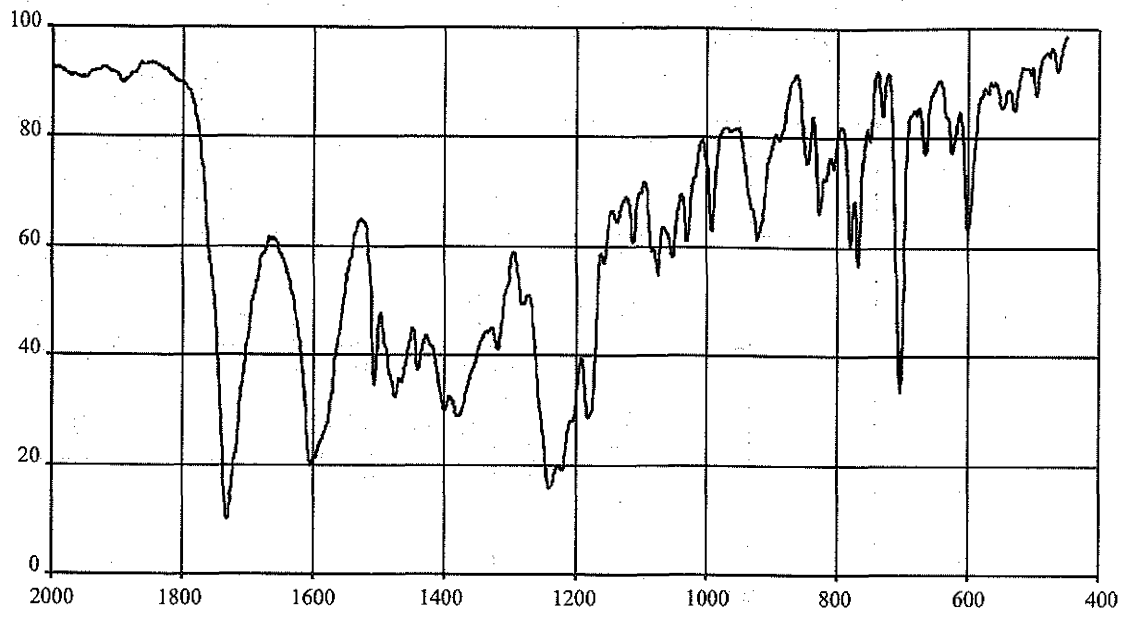
Tacrolimus *FTIR* Phase: KBr disc



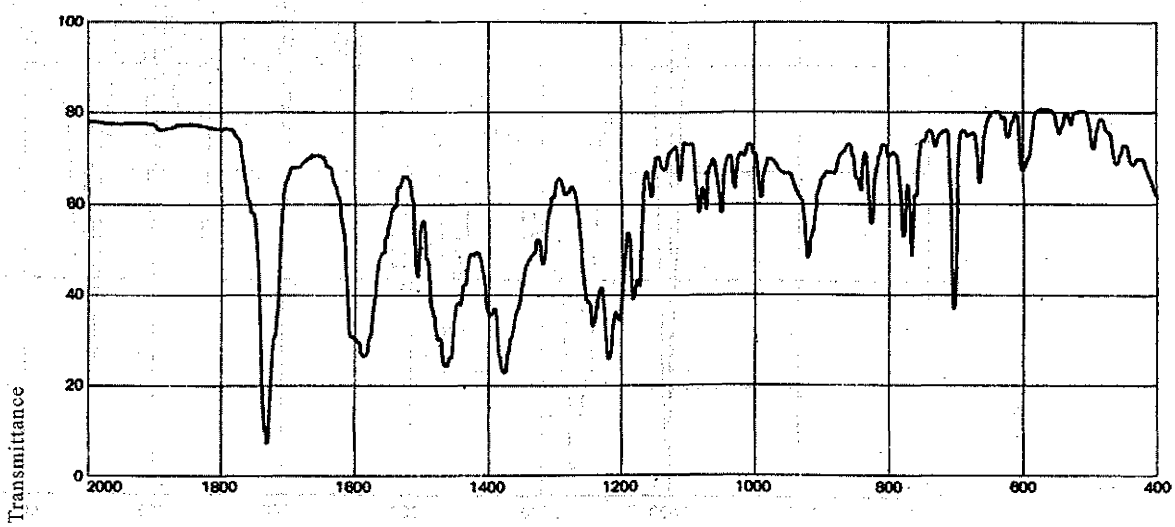
Tadalafil *FTIR* Phase: KBr disc



Tamoxifen *Dispersive IR* Phase: KBr disc



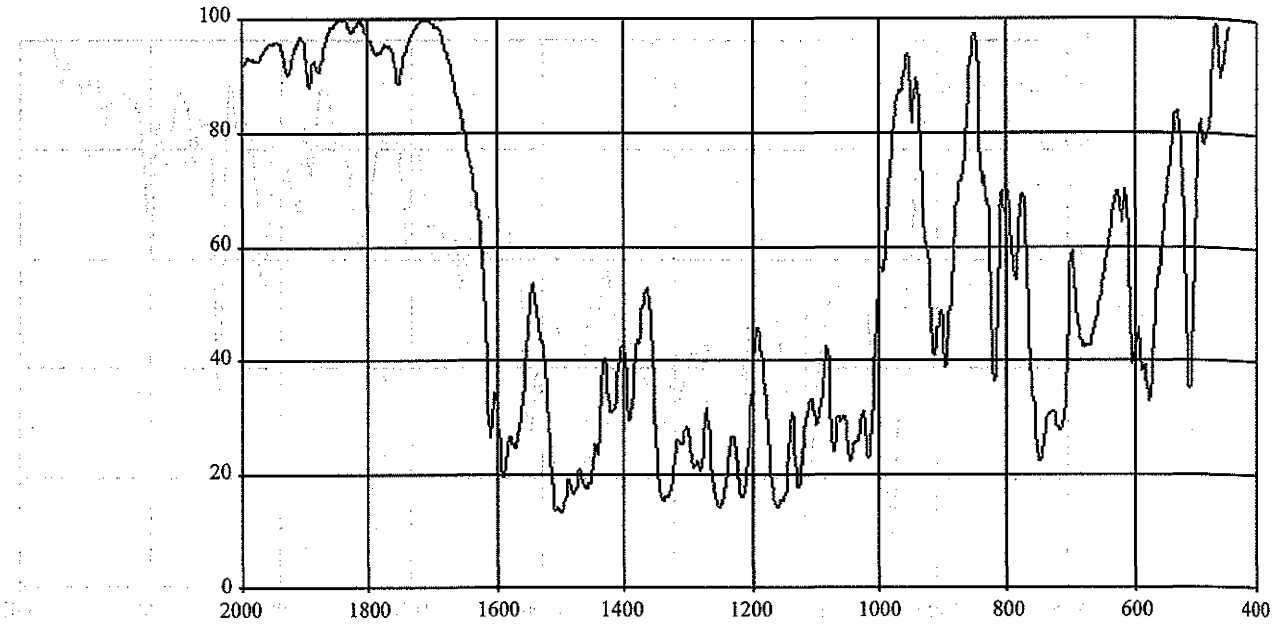
Tamoxifen Citrate *Dispersive IR* Phase: Liquid paraffin mull



Tamsulosin Hydrochloride

FTIR

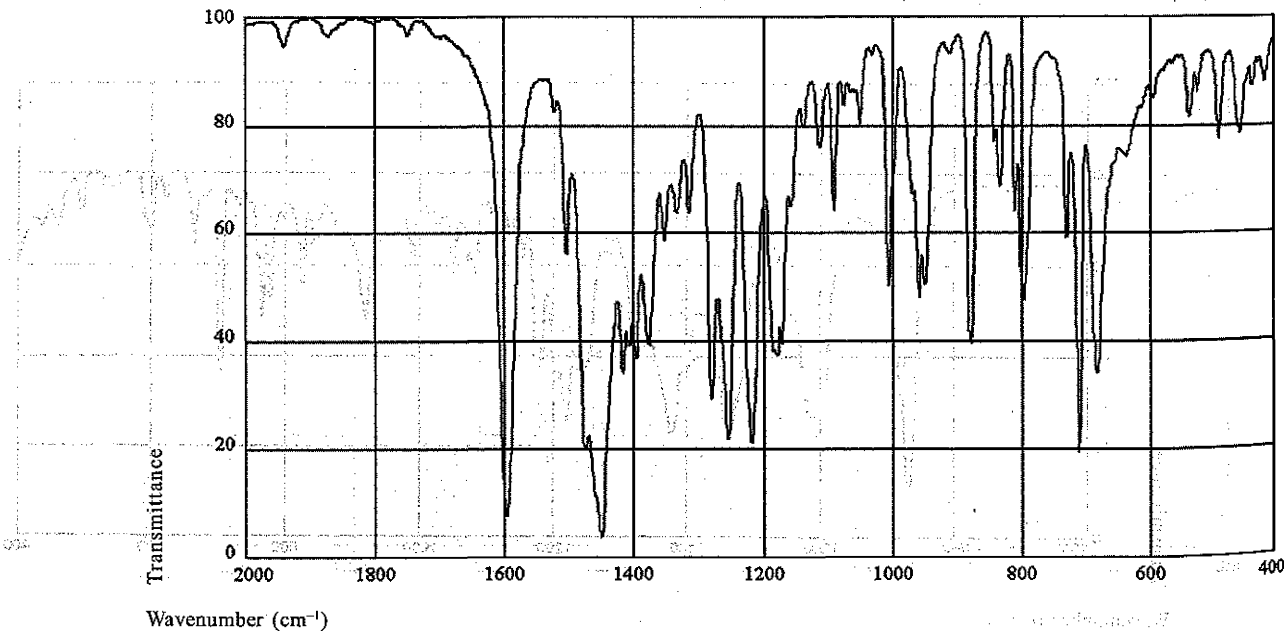
Phase: KBr disc



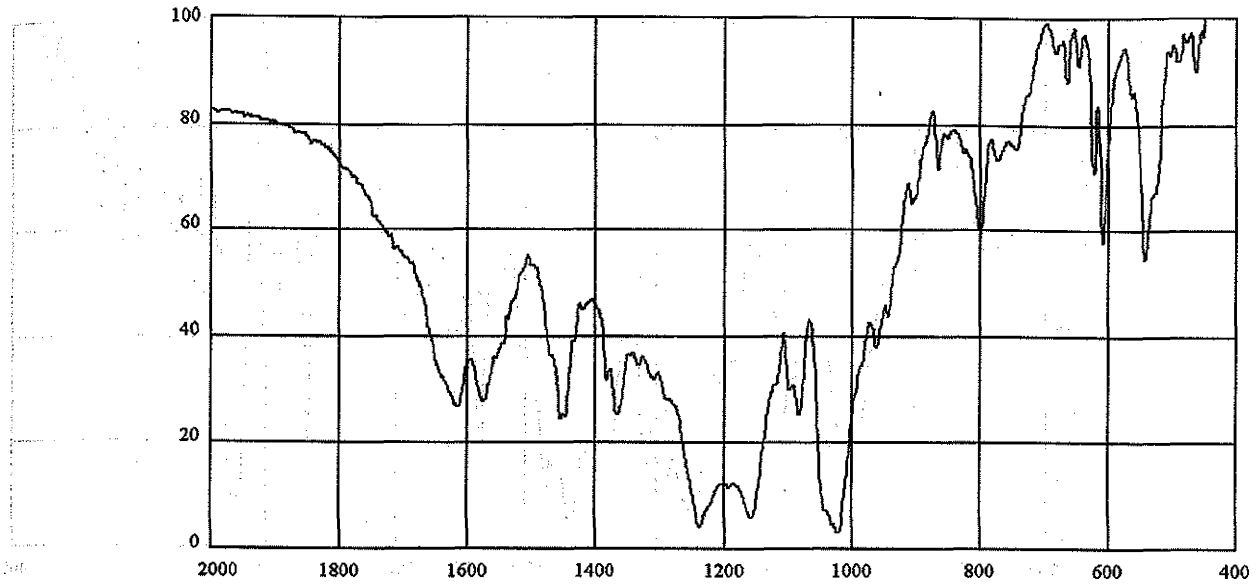
Tapentadol Hydrochloride

FTIR

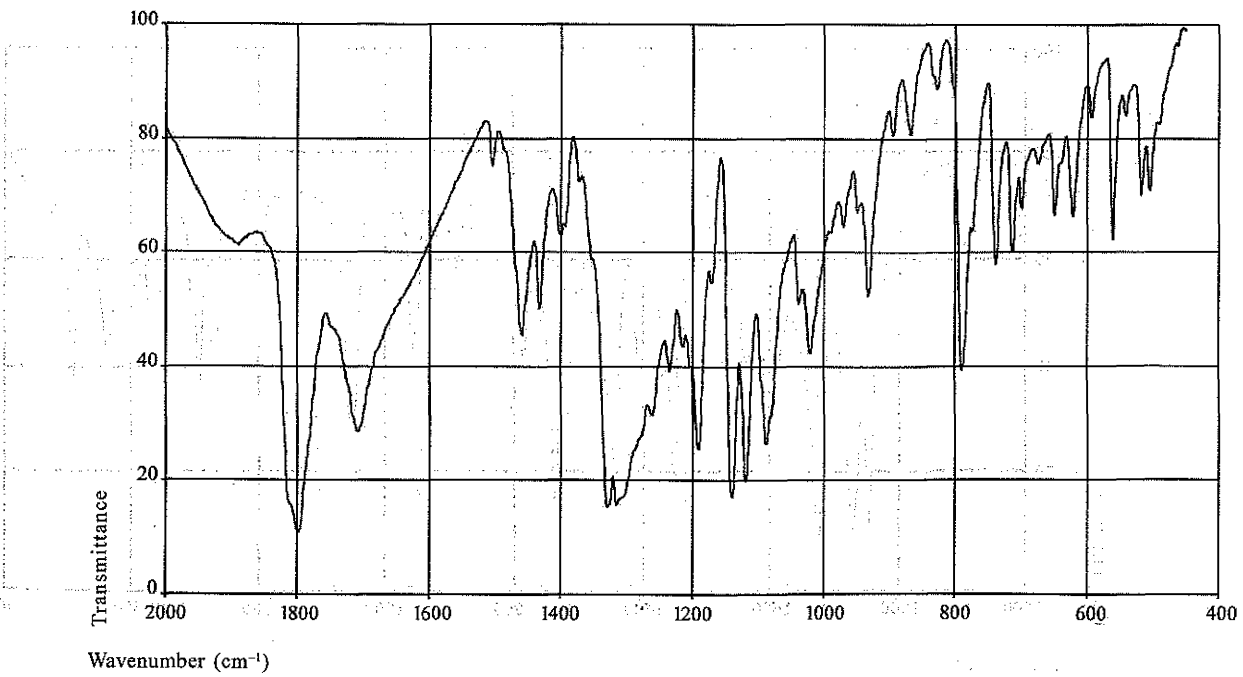
Phase: KBr disc



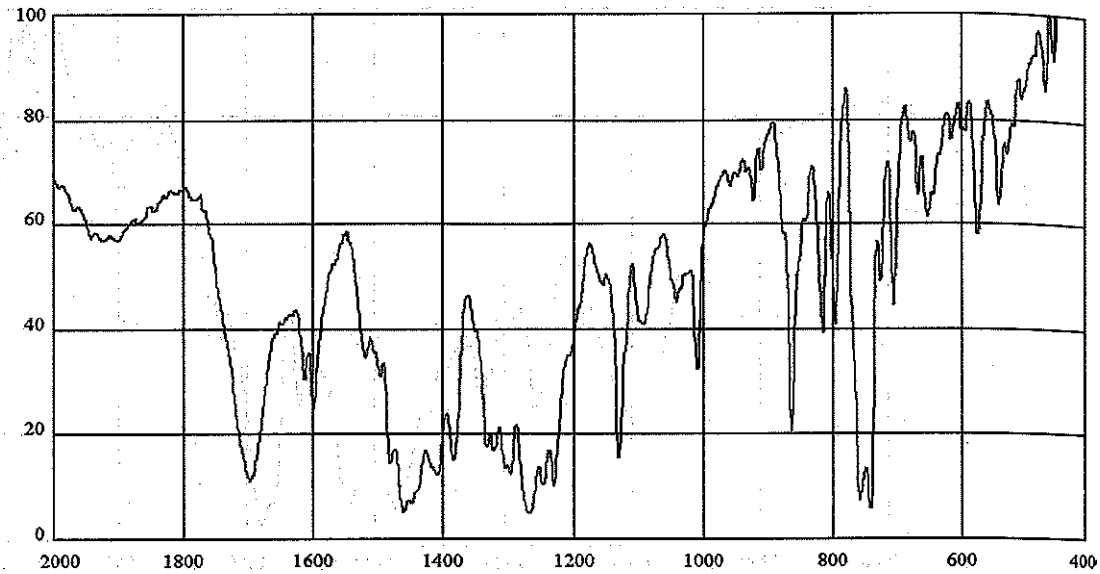
Tauroursodeoxycholic Acid *FTIR* Phase: KBr disc



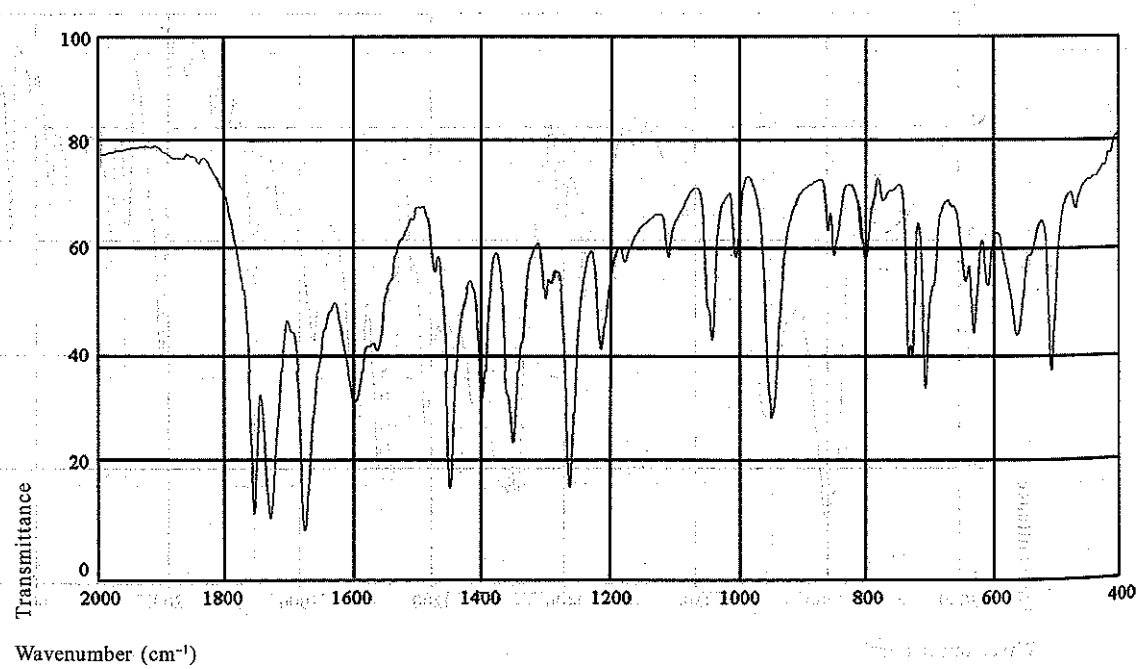
Tazobactam *FTIR* Phase: KBr disc



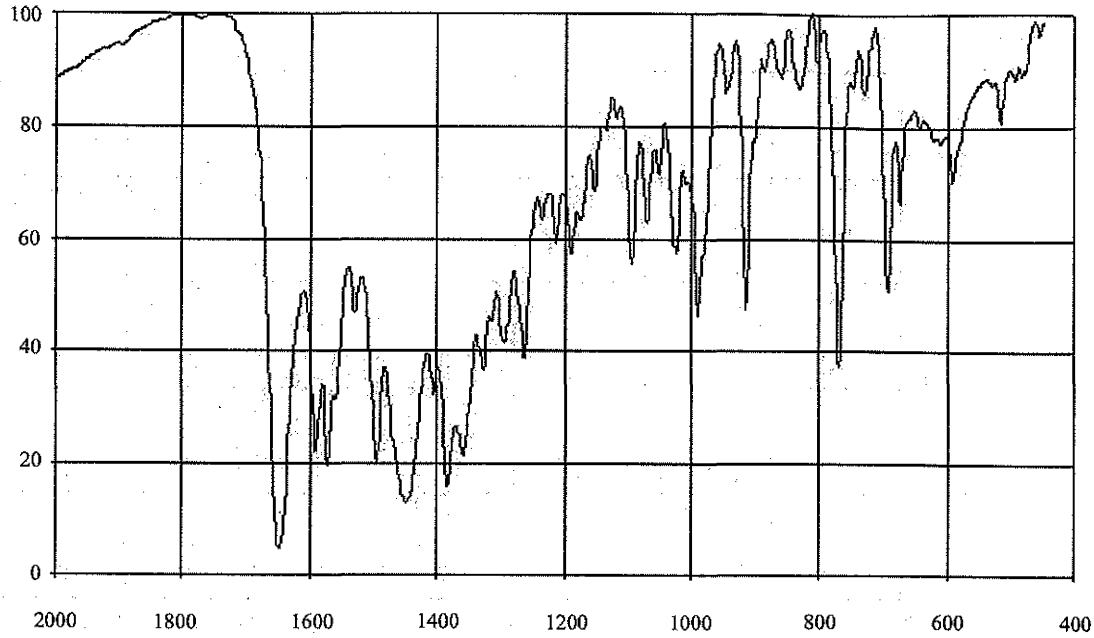
Telmisartan *FTIR* Phase: KBr disc



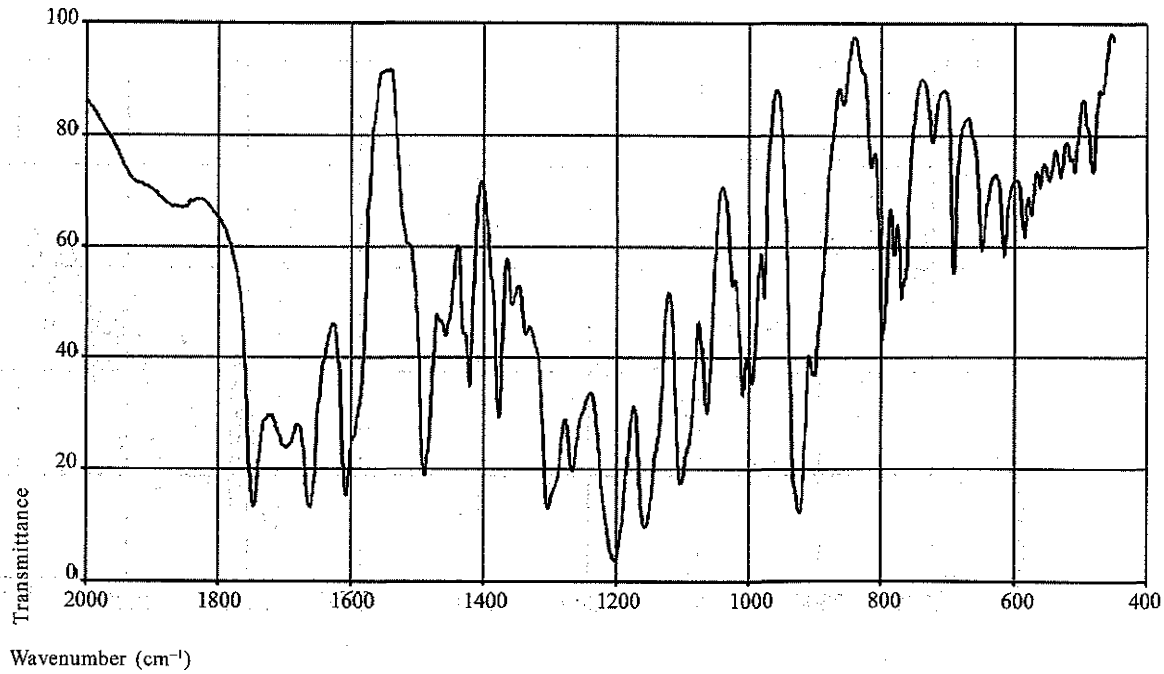
Temozolomide *FTIR* Phase: KBr disc



Teneligliptin Hydrobromide Hydrate *FTIR* Phase: KBr disc



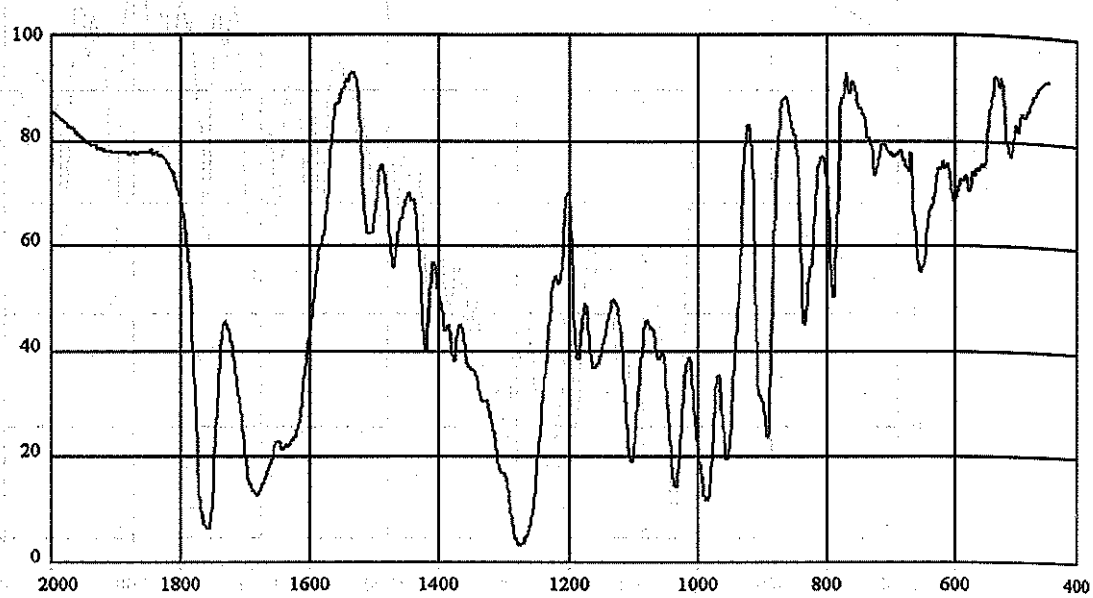
Tenofovir Alafenamide Fumarate *FTIR* Phase: KBr disc



Tenofovir Disoproxil Fumarate

FTIR

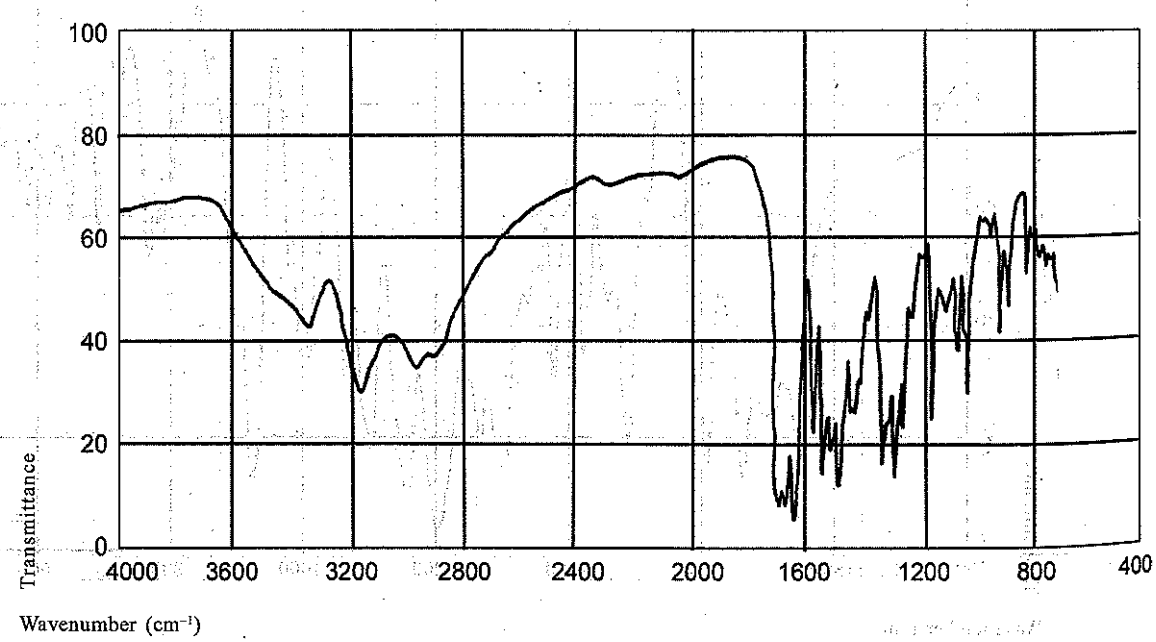
Phase: KBr disc



Terazosin Hydrochloride

FTIR

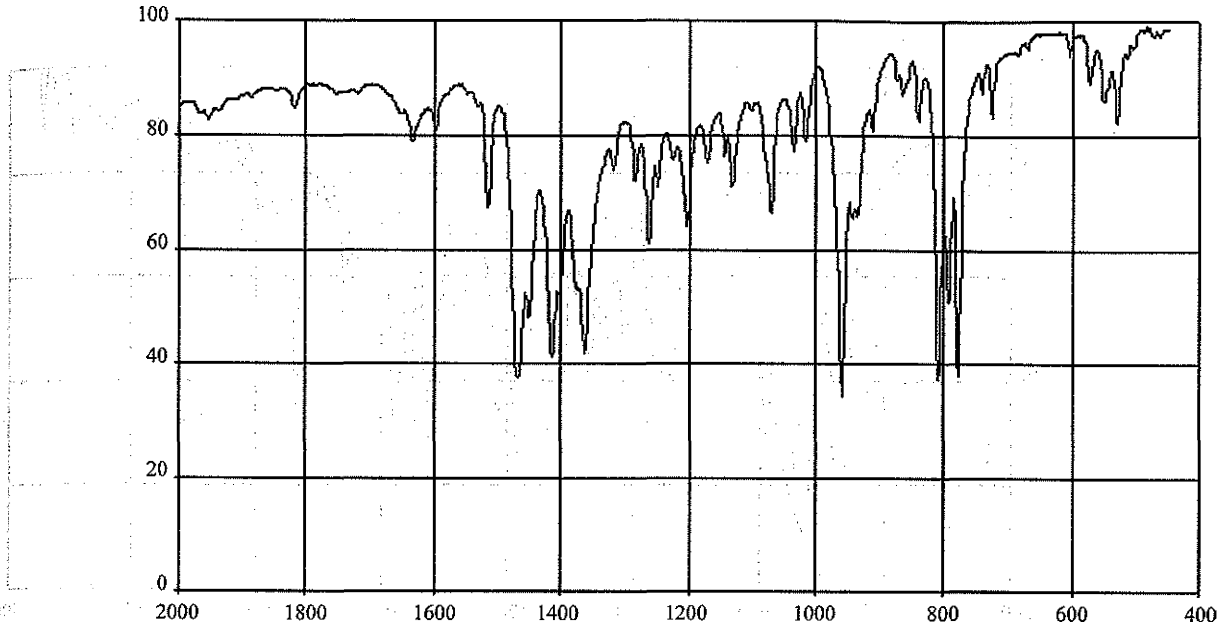
Phase: KBr disc



Terbinafine Hydrochloride

FTIR

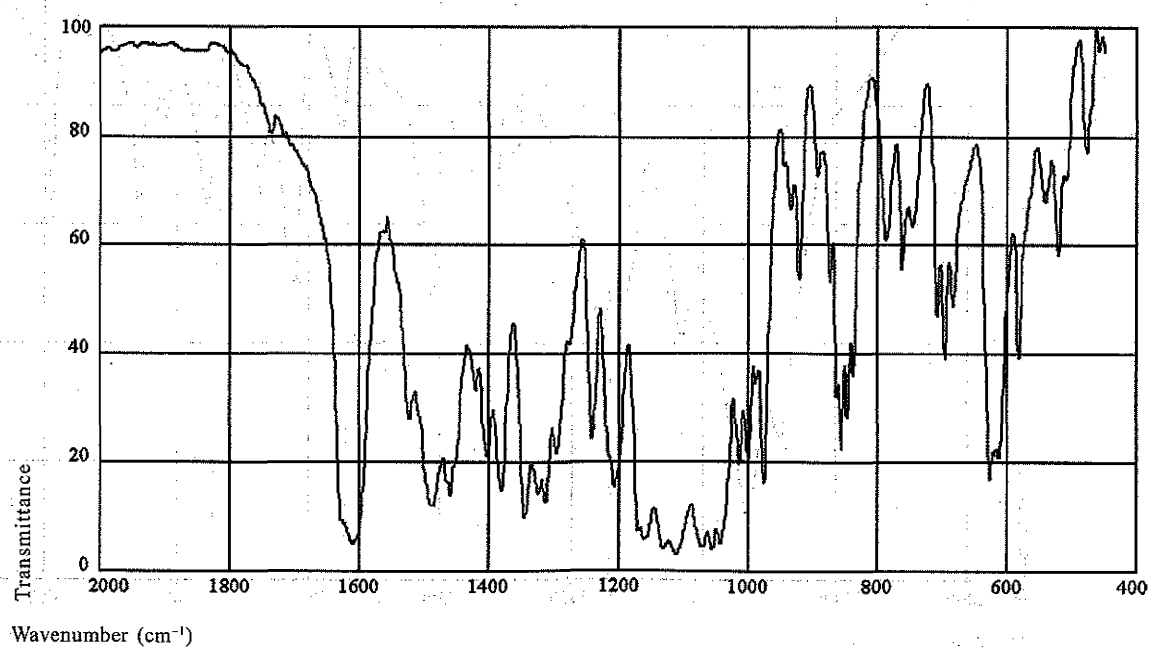
Phase: KBr disc



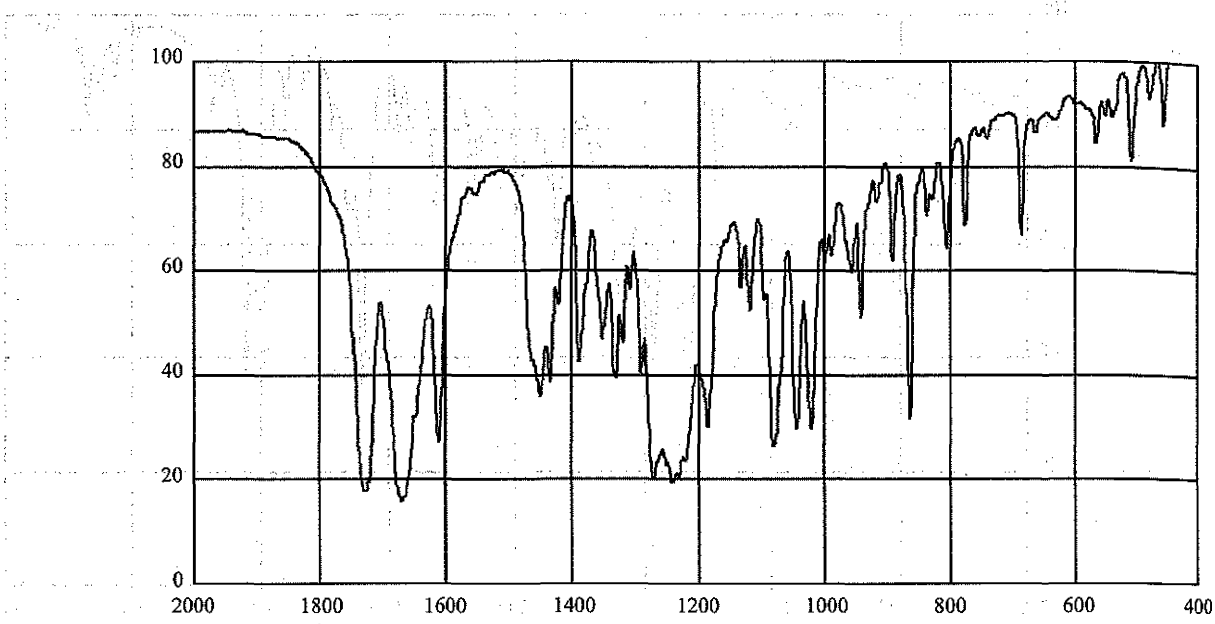
Terbutaline Sulphate

FTIR

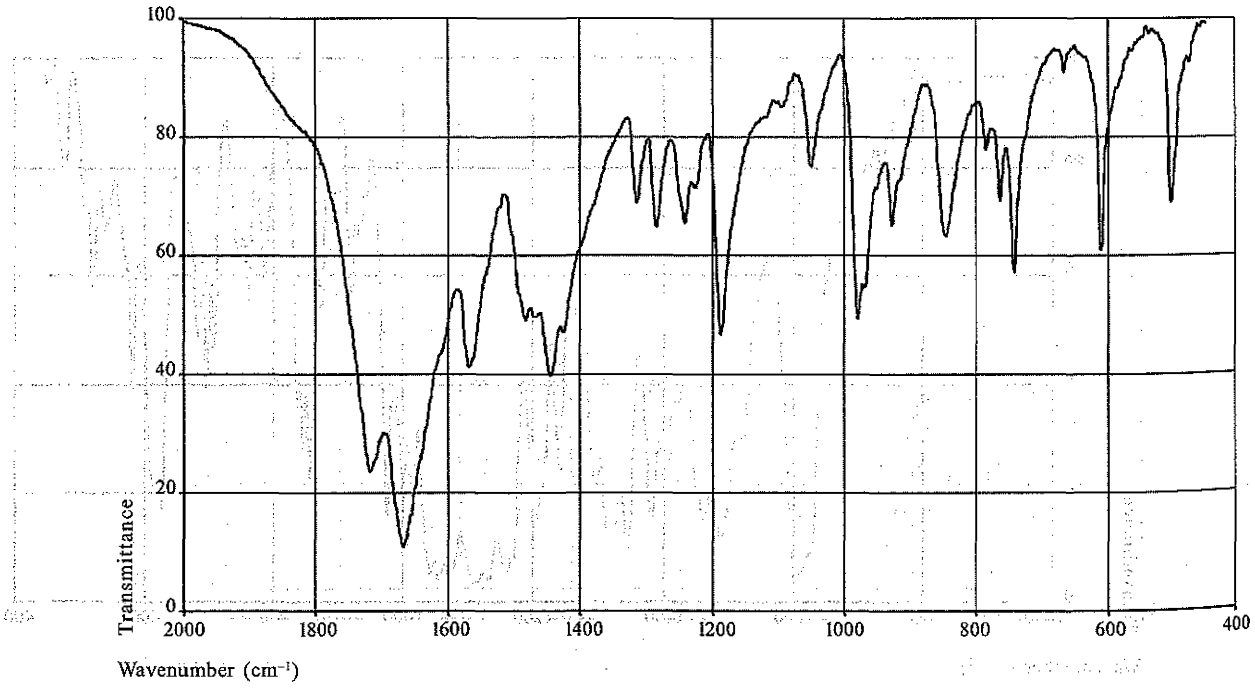
Phase: KBr disc



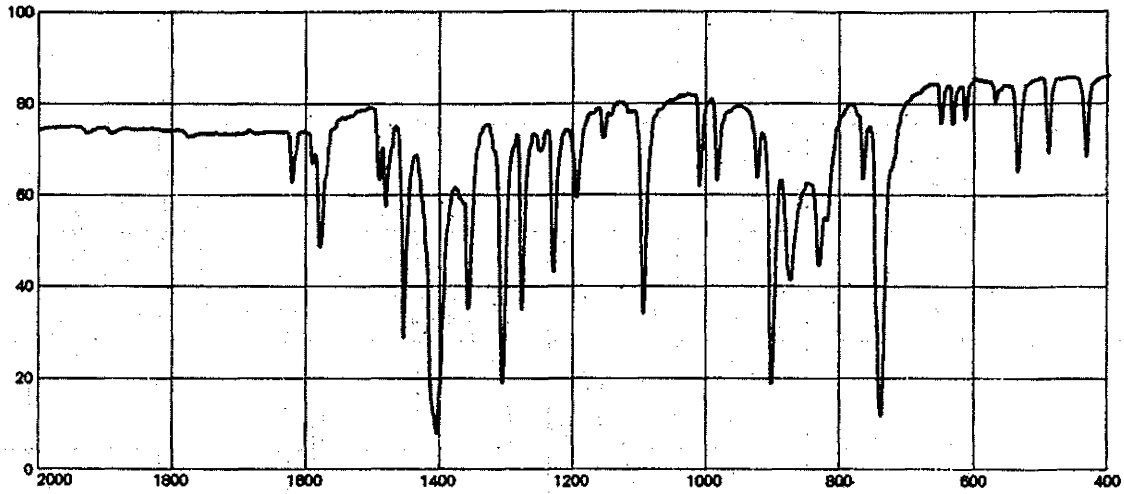
Testosterone Propionate *FTIR* Phase: KBr disc



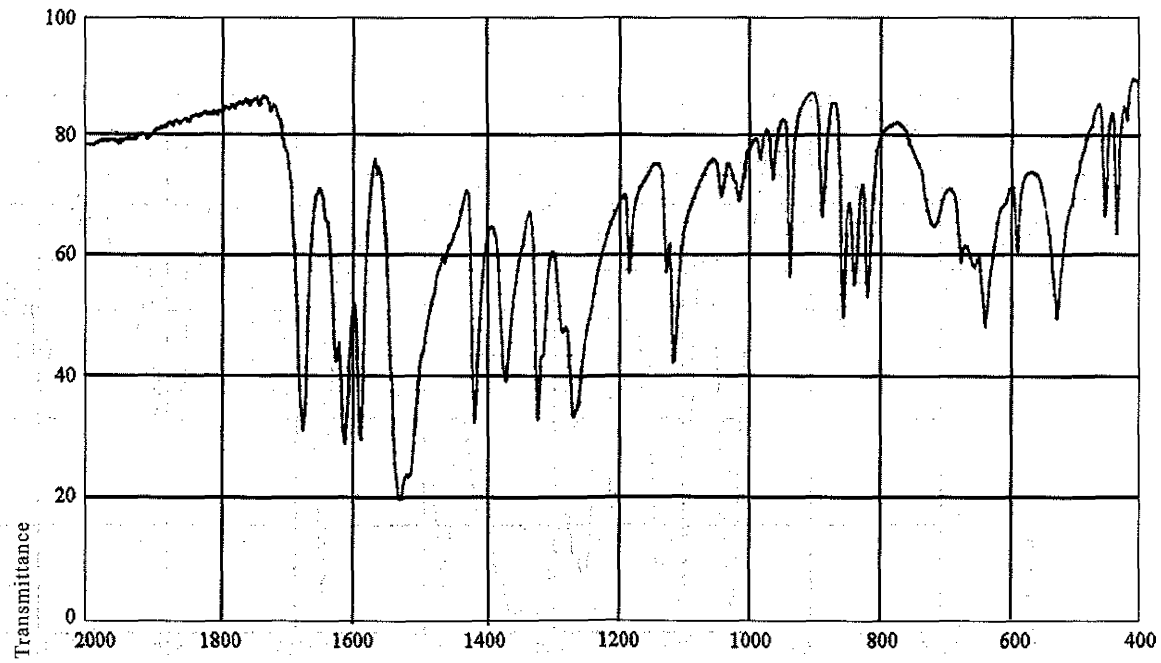
Theophylline *FTIR* Phase: KBr disc



Thiabendazole *Dispersive IR* Phase: KBr disc



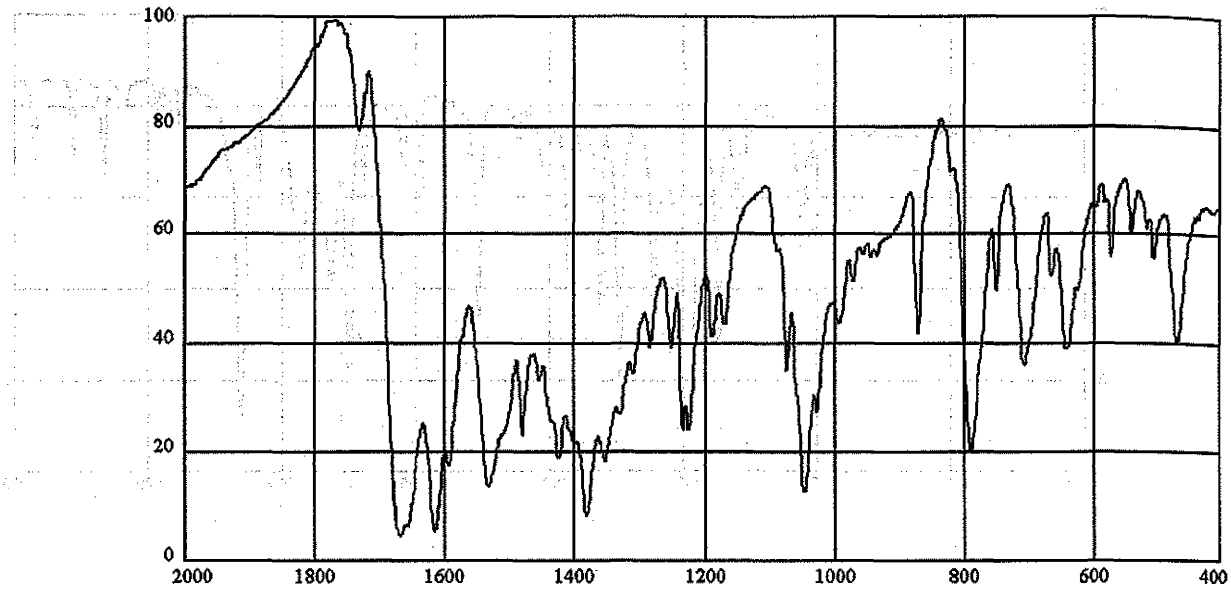
Thiacetazone *FTIR* Phase: KBr disc



Thiamine Hydrochloride

FTIR

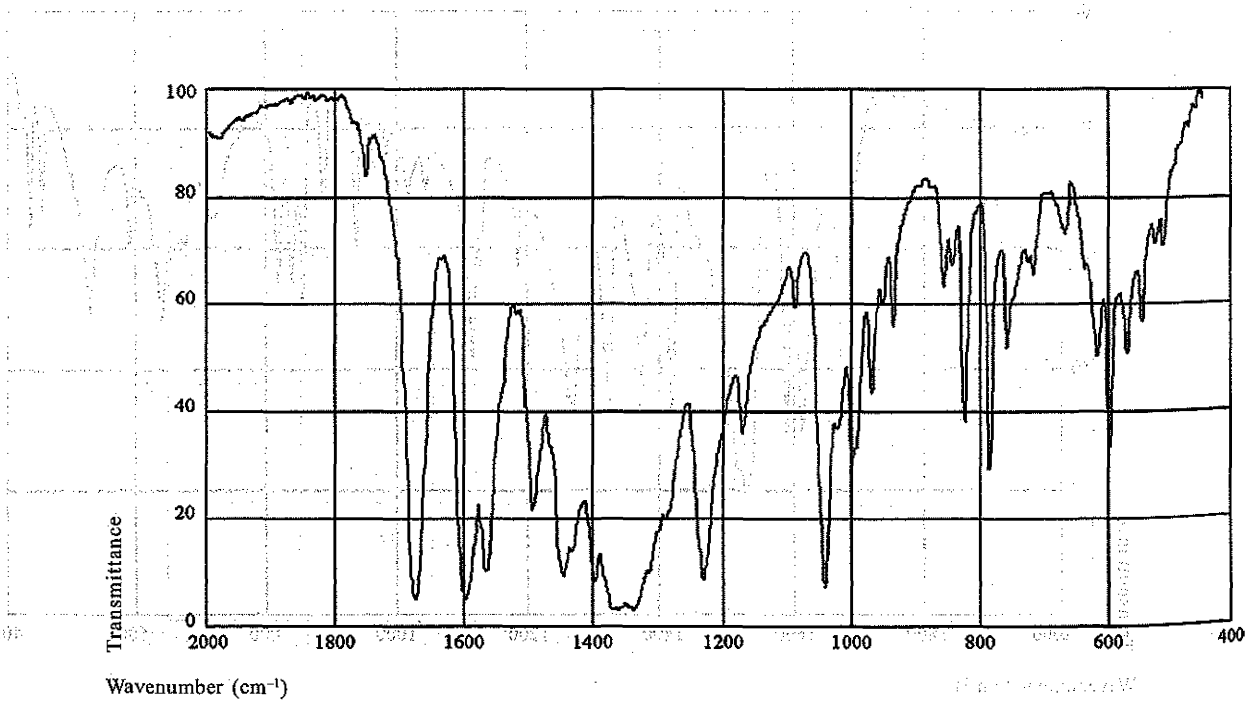
Phase: KBr disc



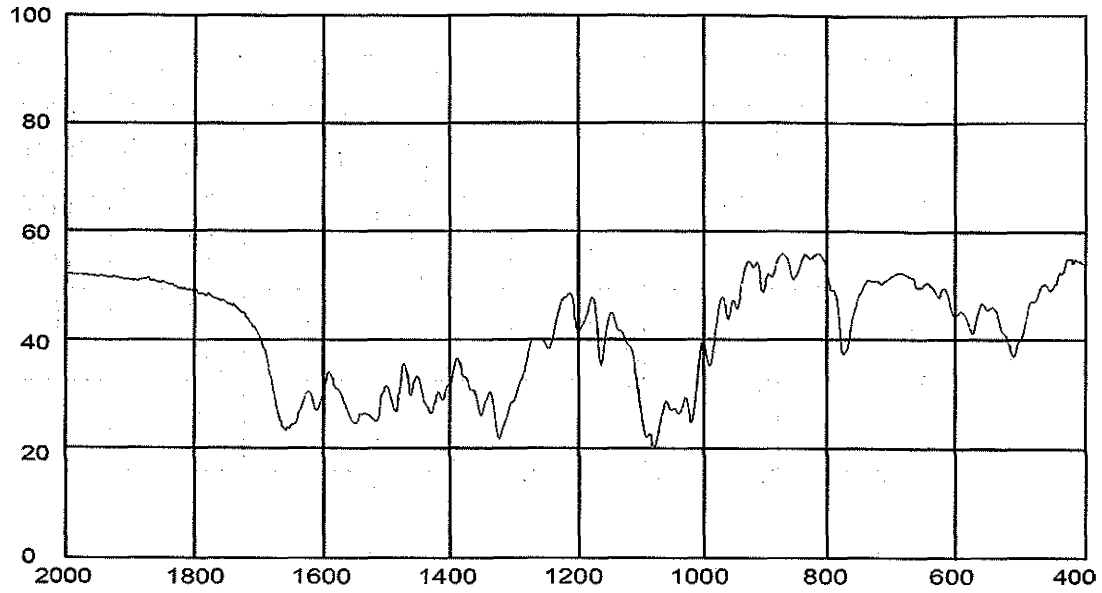
Thiamine Mononitrate

FTIR

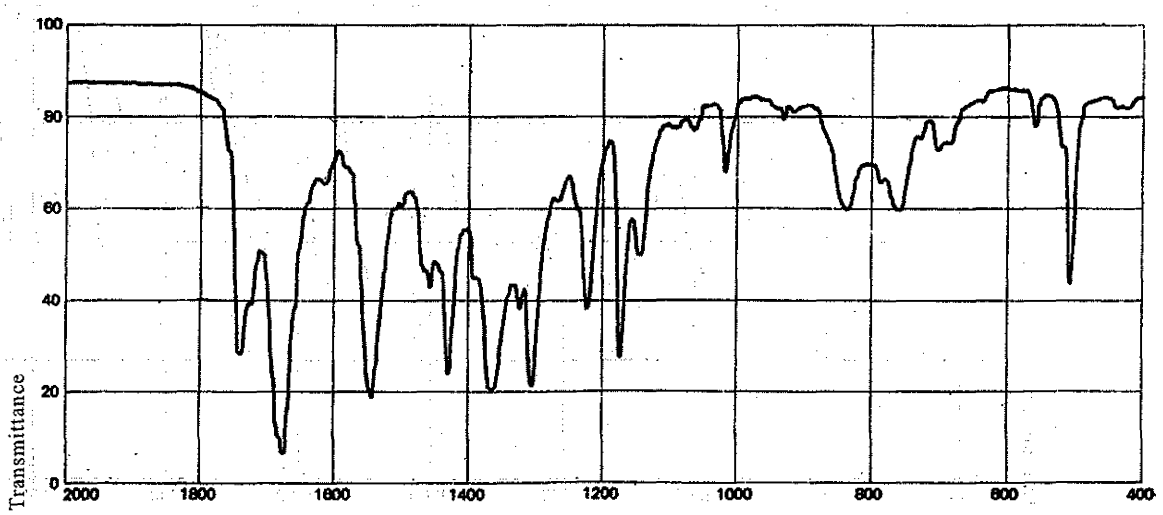
Phase: KBr disc



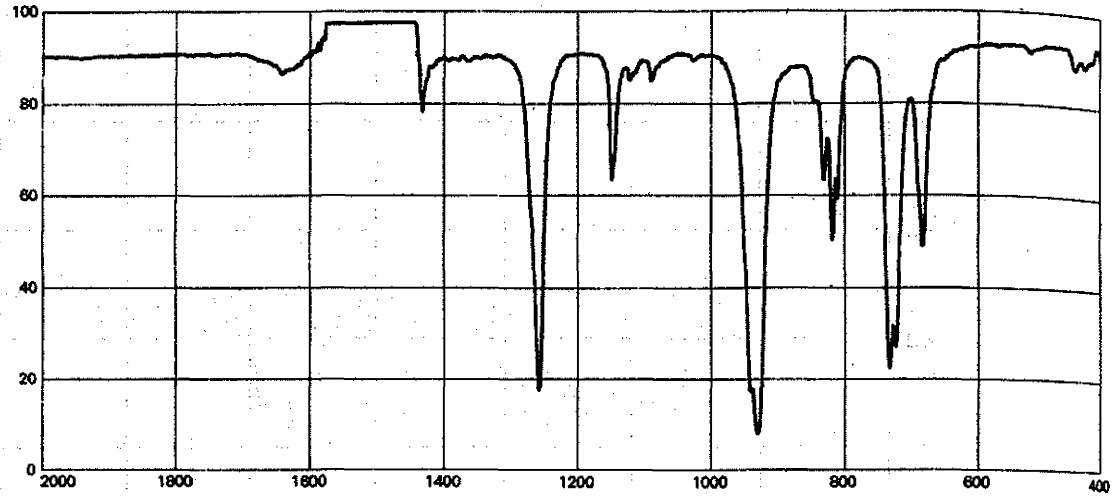
Thiocolchicoside *Dispersive IR* Phase: KBr disc



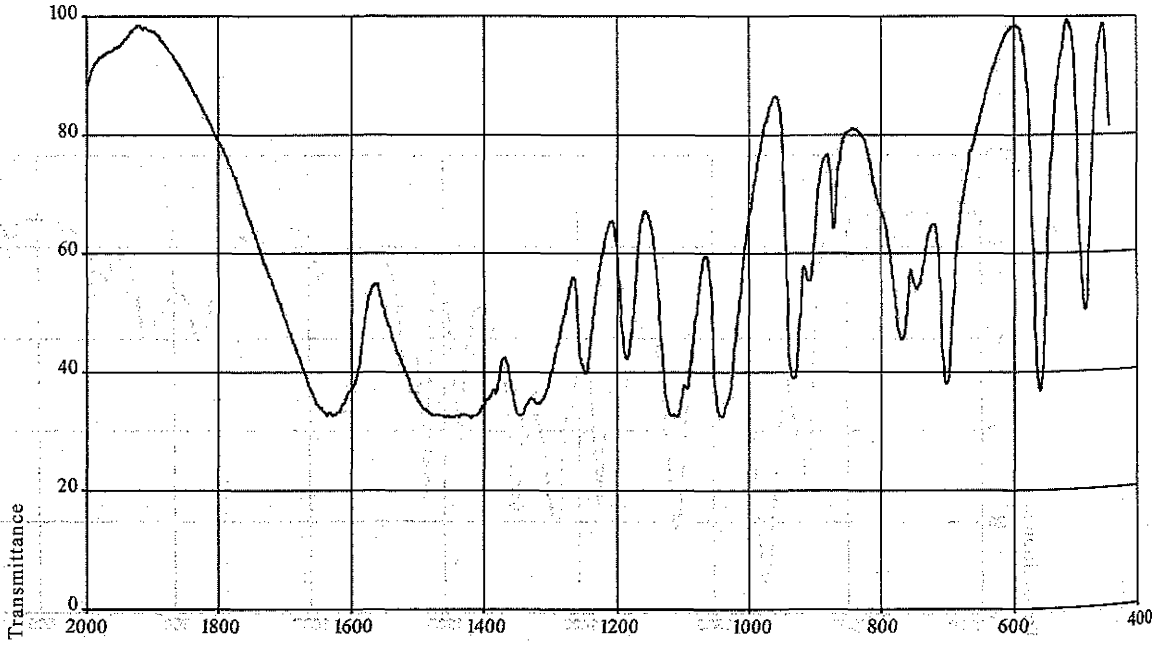
Thiopentone *Dispersive IR* Phase: KBr disc



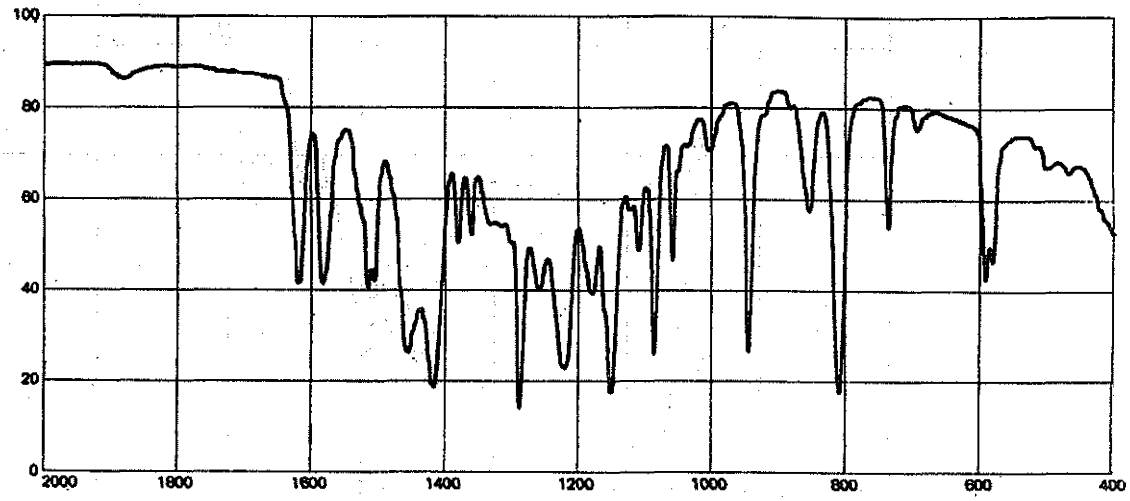
Thiotepa *Dispersive IR* Phase: 2% w/v solution in CS_2 Thickness 0.1 mm



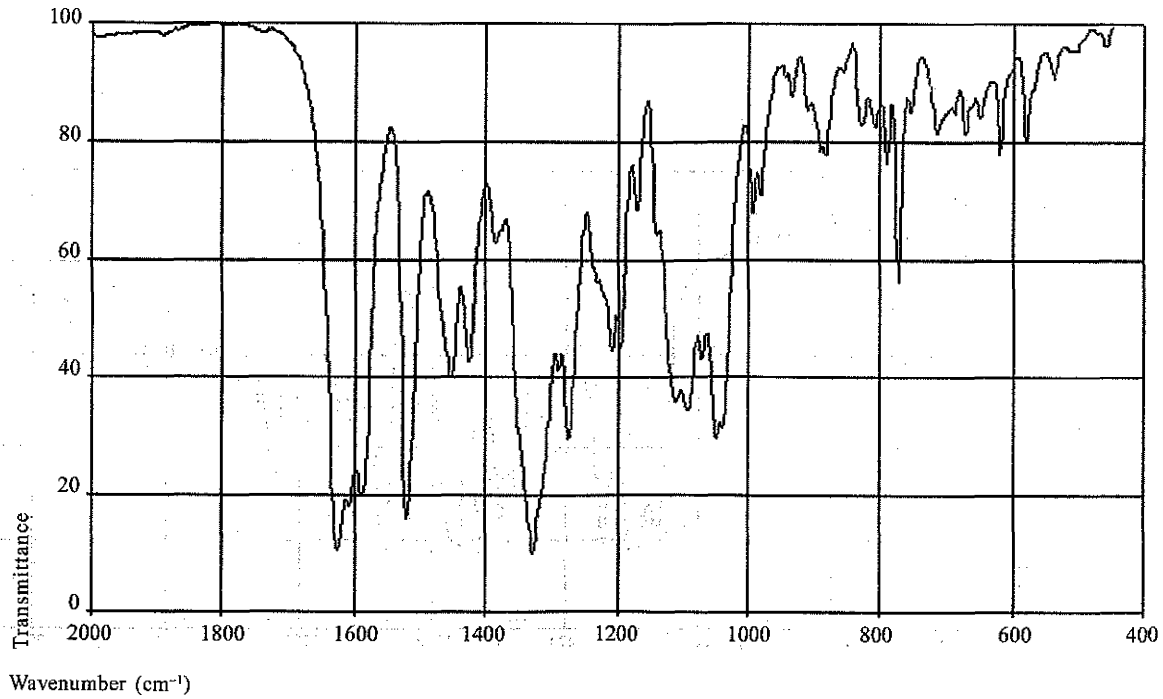
Threonine *Dispersive IR* Phase: Thin film



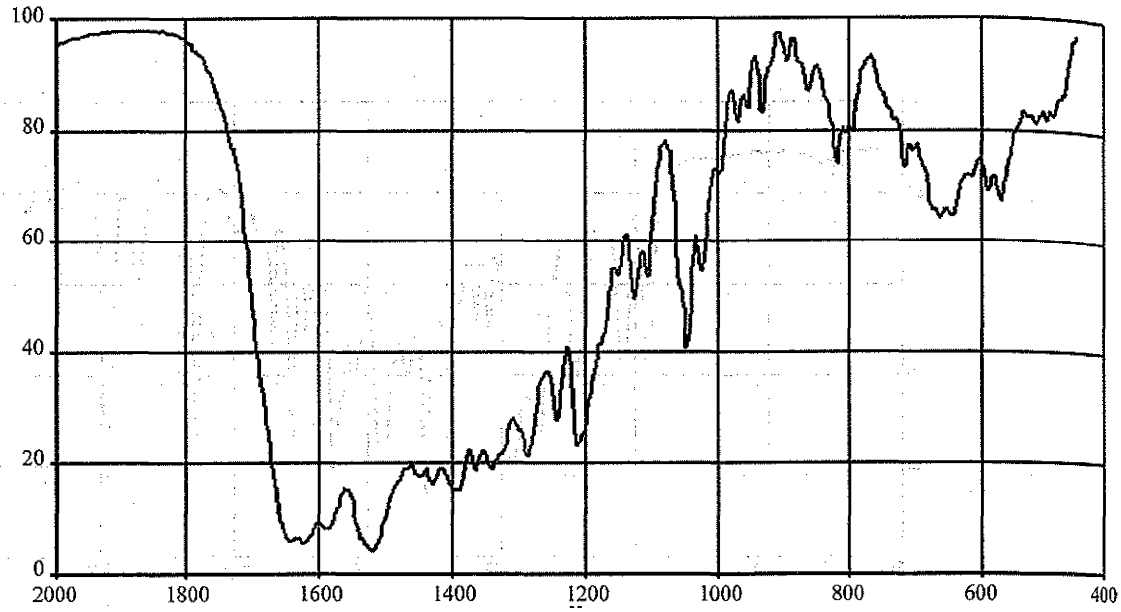
Thymol *Dispersive IR* Phase: Thin film



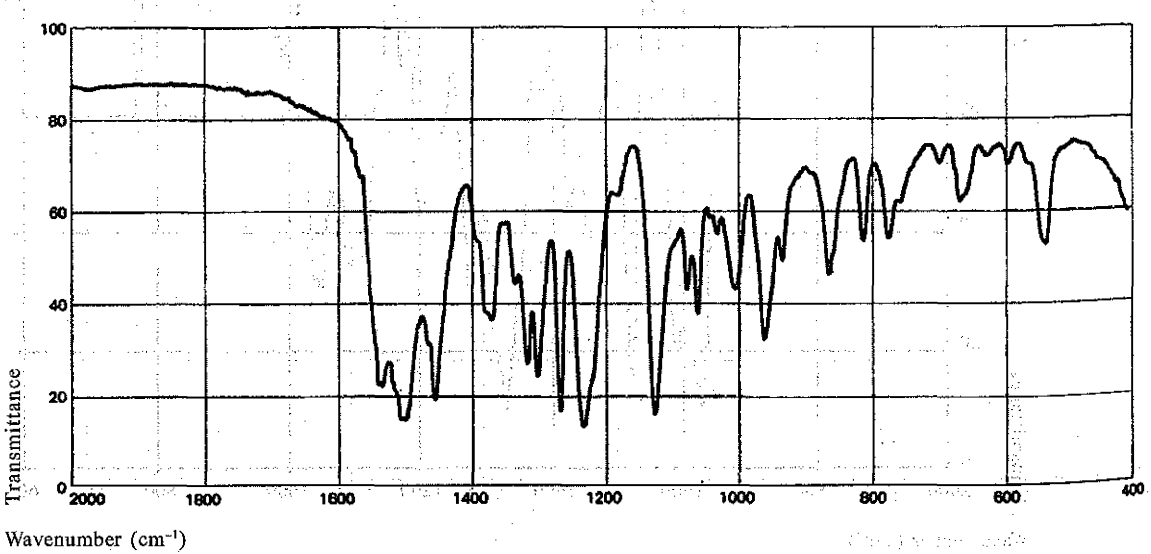
Ticagrelor *FTIR* Phase: KBr disc



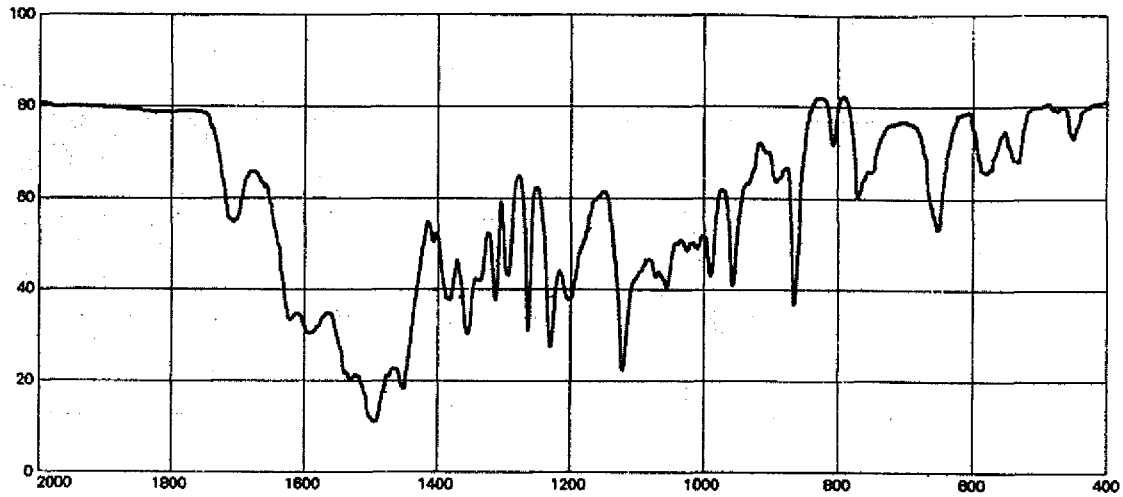
Tigecycline *FTIR* Phase: KBr disc



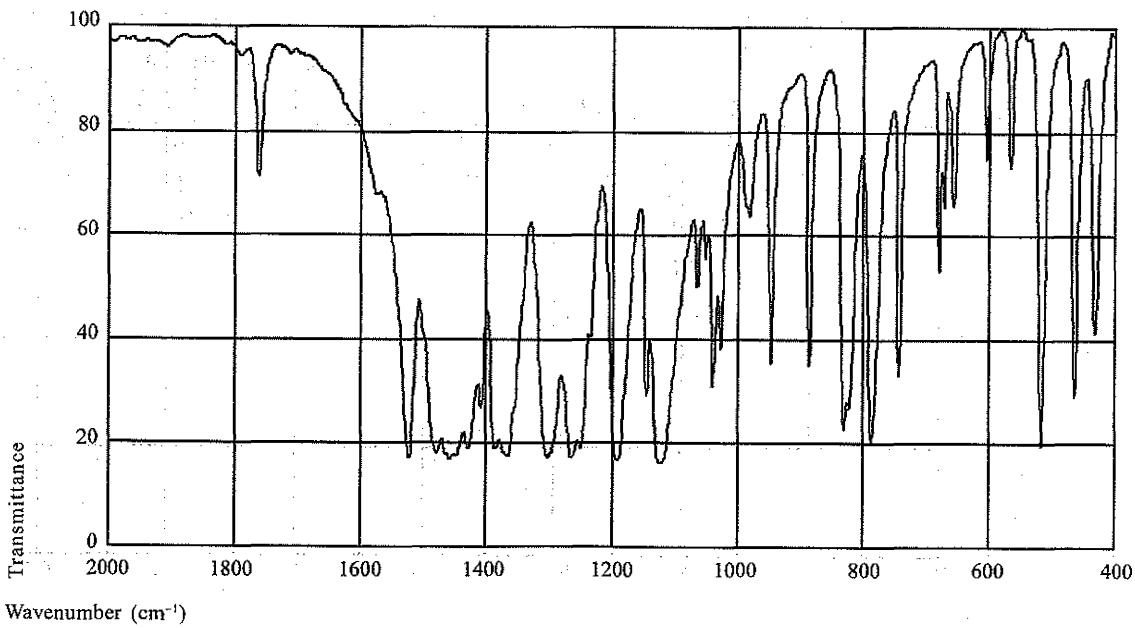
Timolol *Dispersive IR* Phase: Thin film



Timolol Maleate *Dispersive IR* Phase: KBr disc



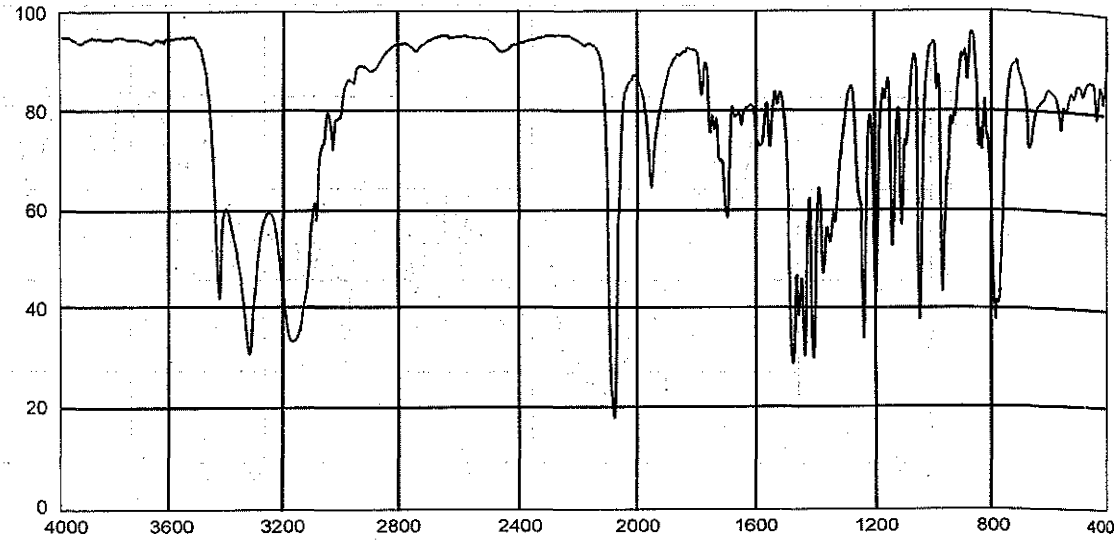
Tinidazole *FTIR* Phase: KBr disc



Tiotropium Bromide

FTIR

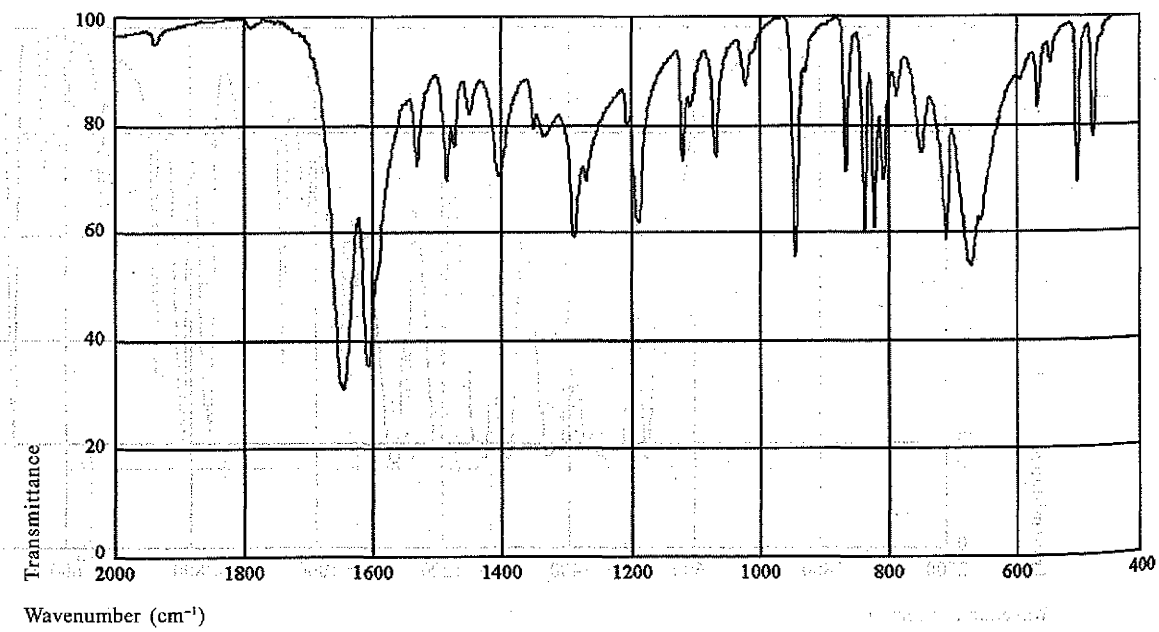
Phase: KBr disc



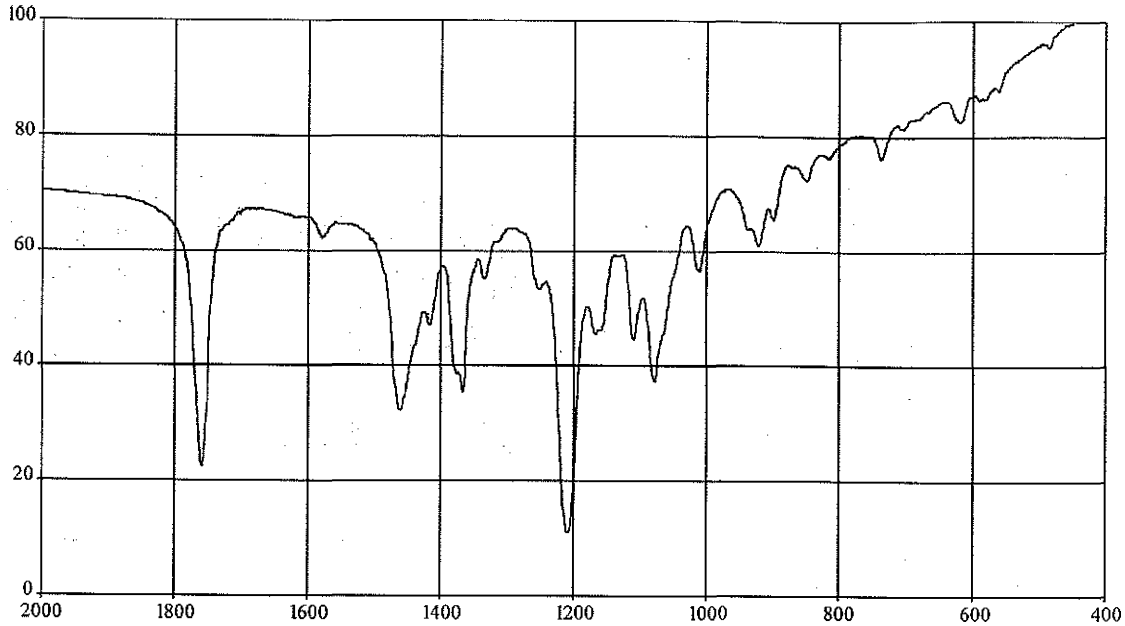
Tizanidine Hydrochloride

FTIR

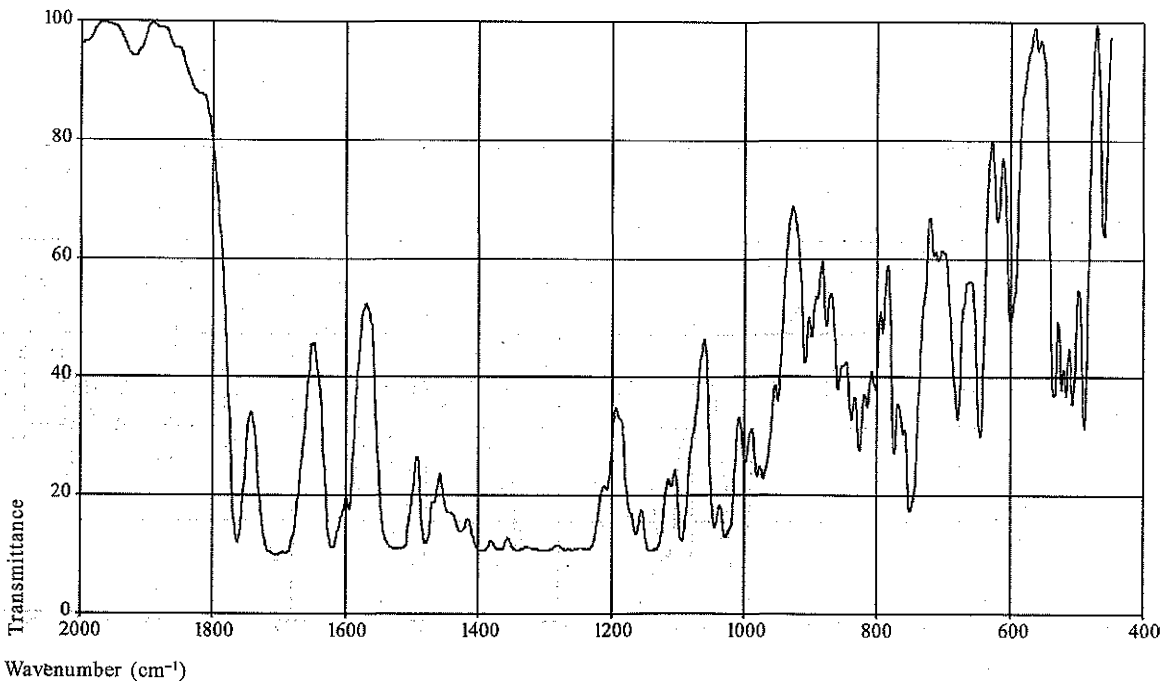
Phase: KBr disc



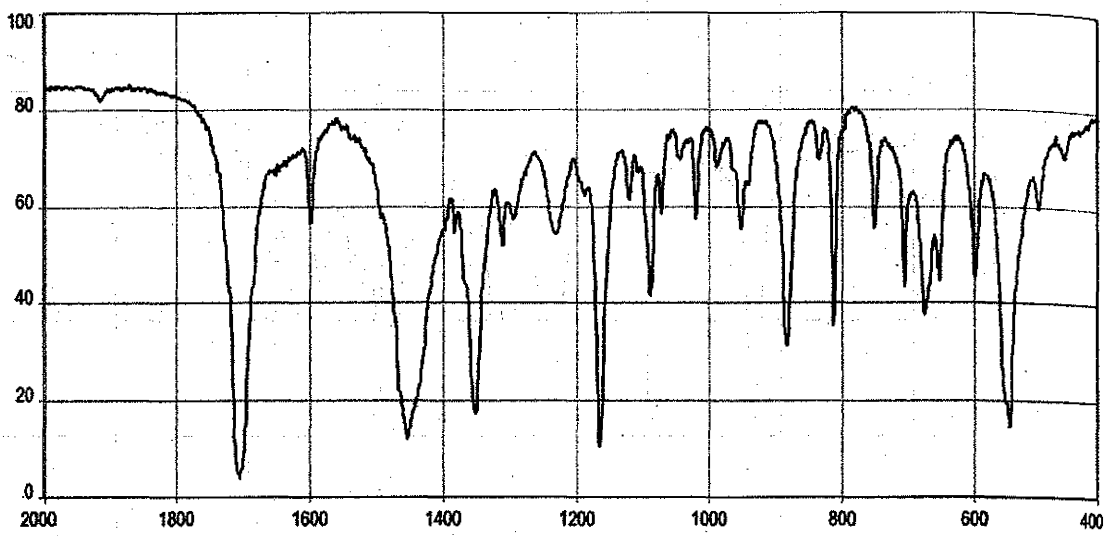
Alpha Tocopheryl Acetate *FTIR* Phase: KBr disc



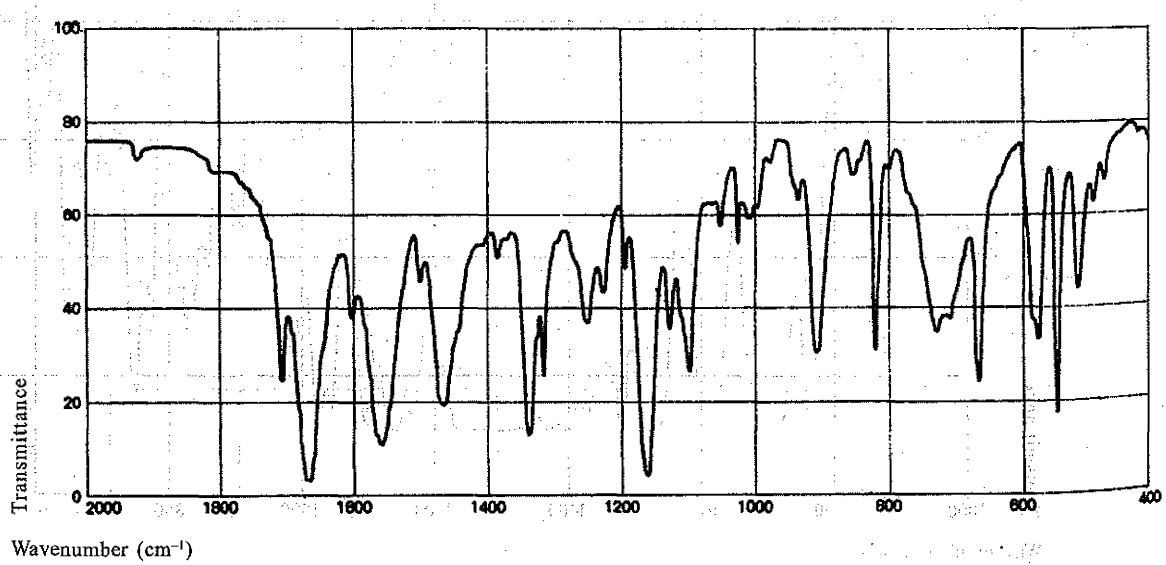
Tofacitinib Citrate *Dispersive IR* Phase: KBr disc



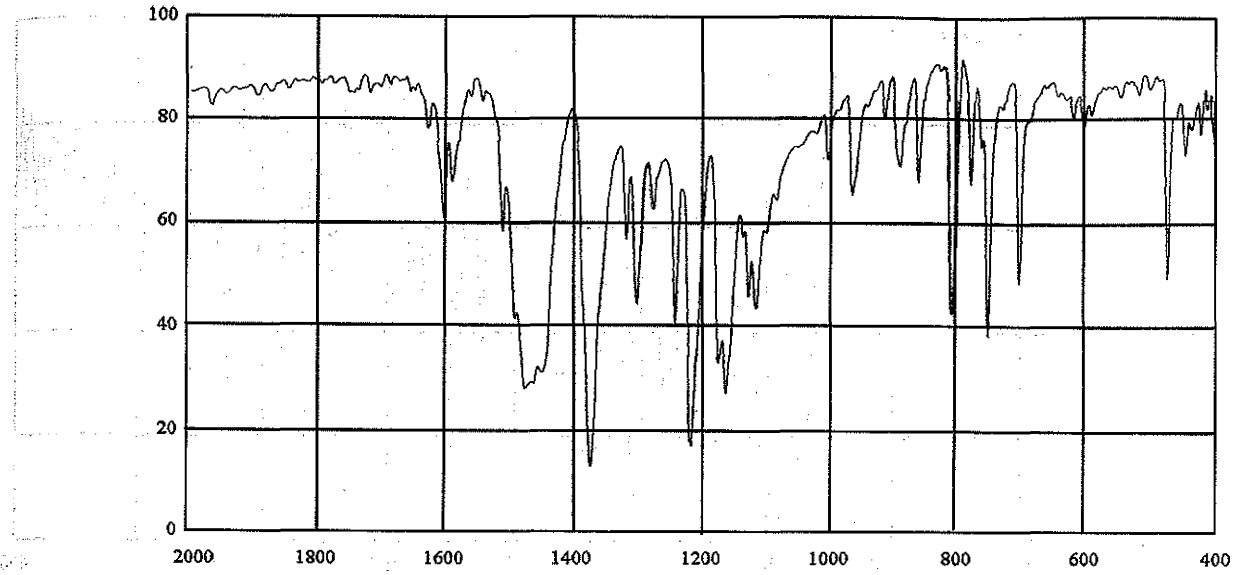
Tolazamide *Dispersive IR* Phase: KBr disc



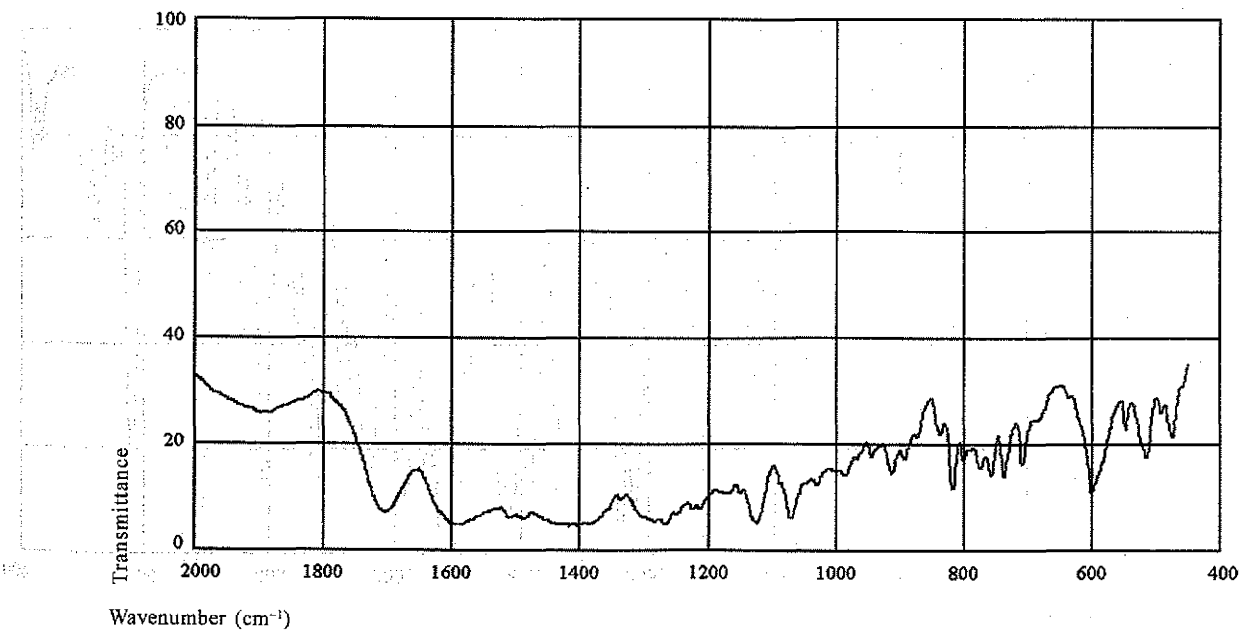
Tolbutamide *Dispersive IR* Phase: KBr disc



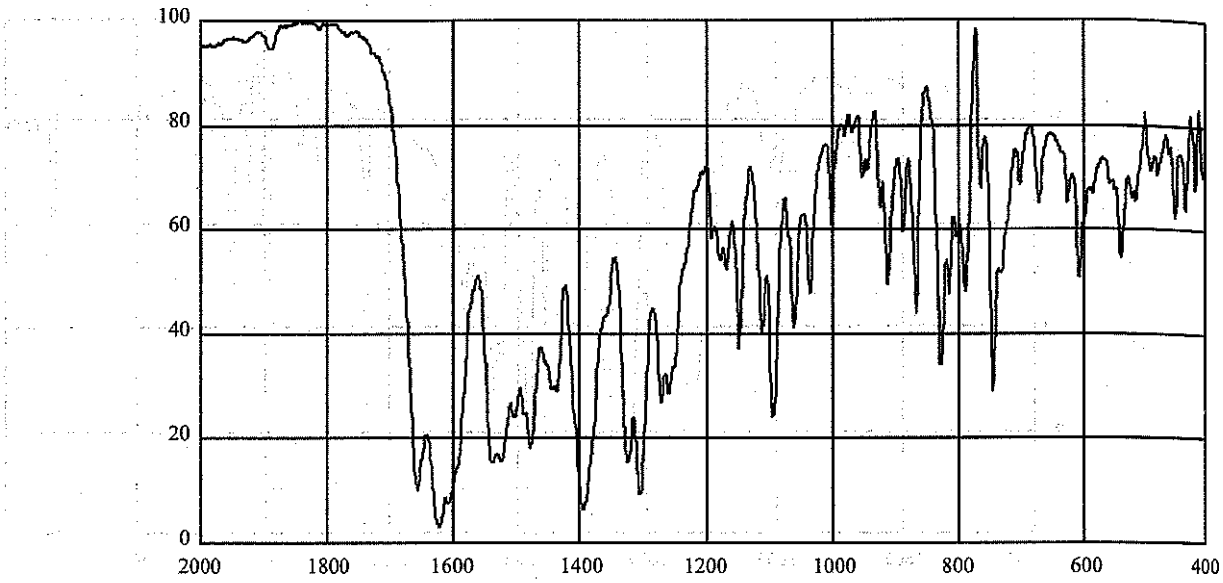
Tolnaftate *Dispersive IR* Phase: KBr disc



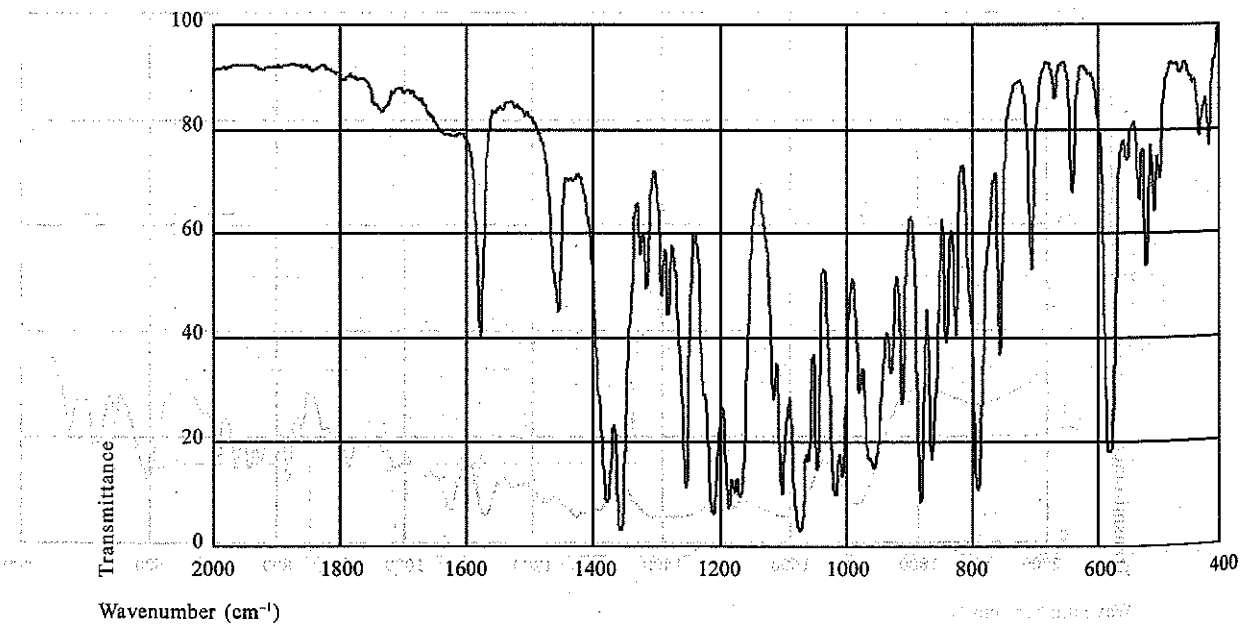
Tolterodine Tartrate *FTIR* Phase: KBr disc



Tolvaptan *FTIR* Phase: KBr disc



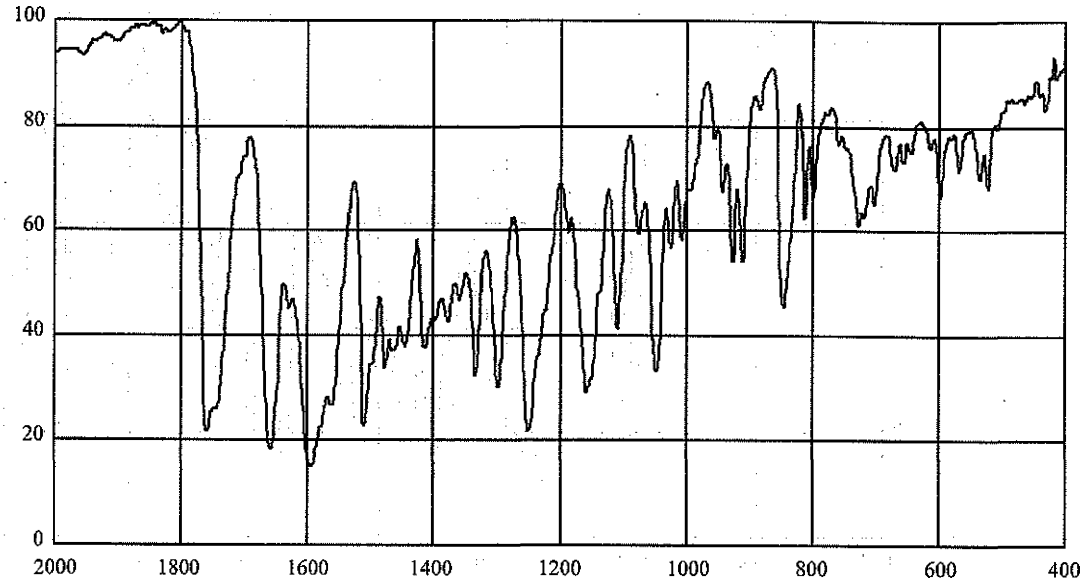
Topiramate *FTIR* Phase: KBr disc



Topotecan Hydrochloride

FTIR

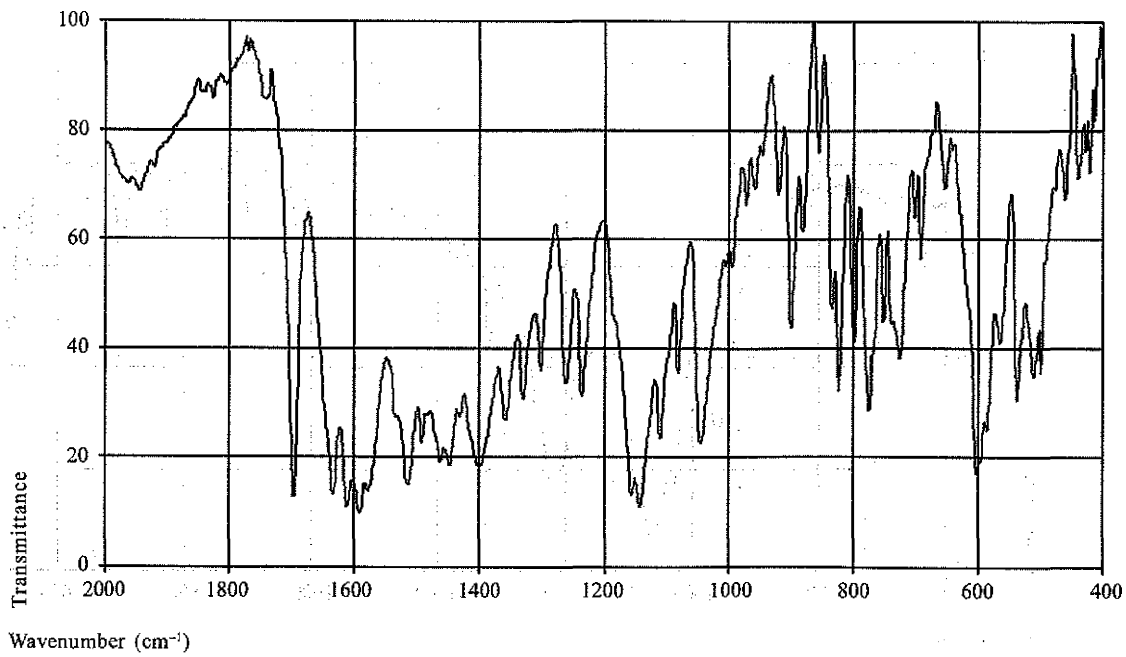
Phase: KBr disc



Torsemide

FTIR

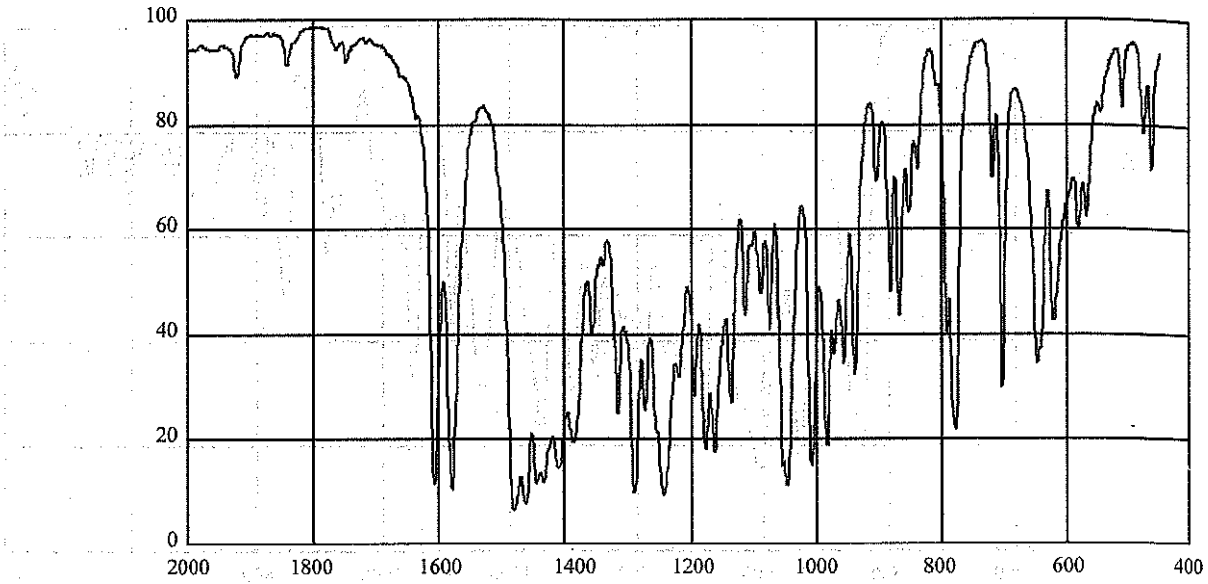
Phase: KBr disc



Tramadol Hydrochloride

FTIR

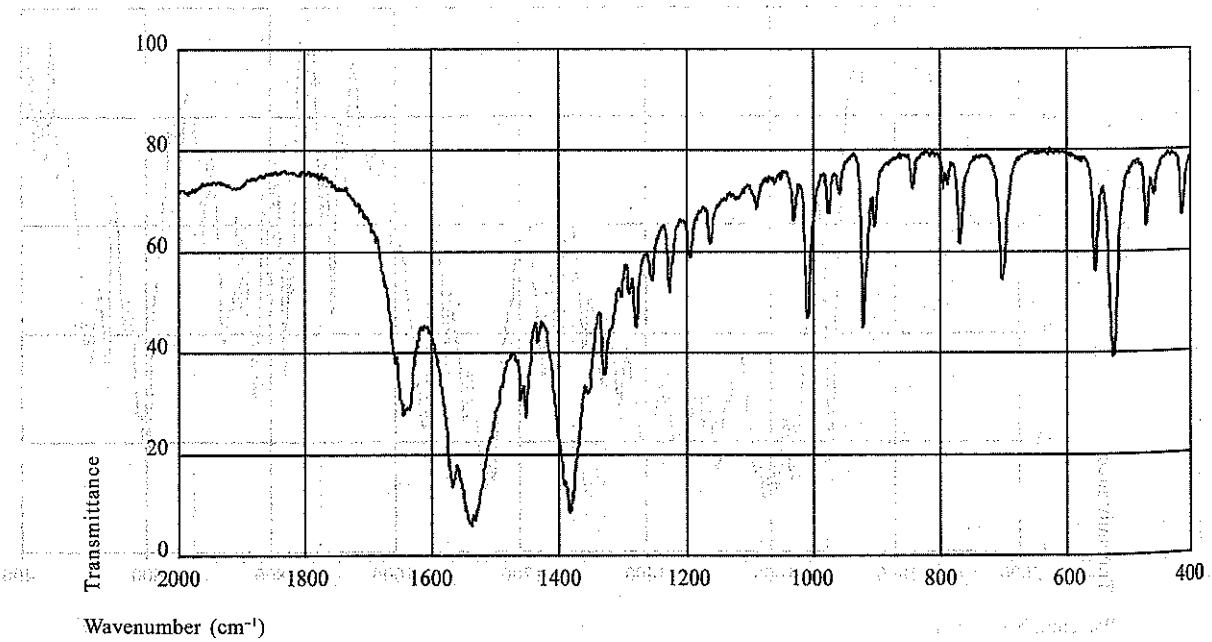
Phase: KBr disc



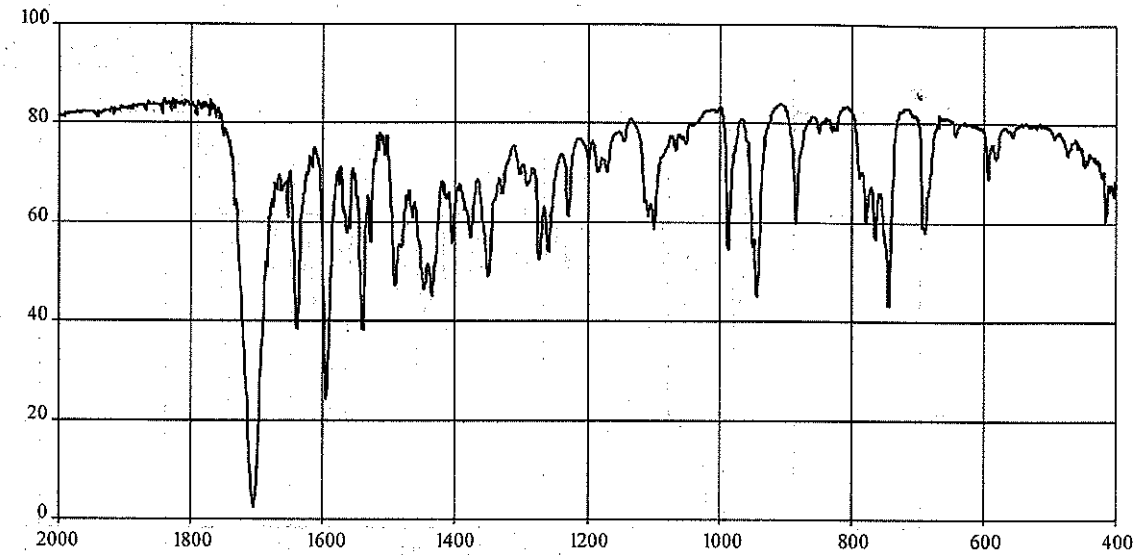
Tranexamic Acid

FTIR

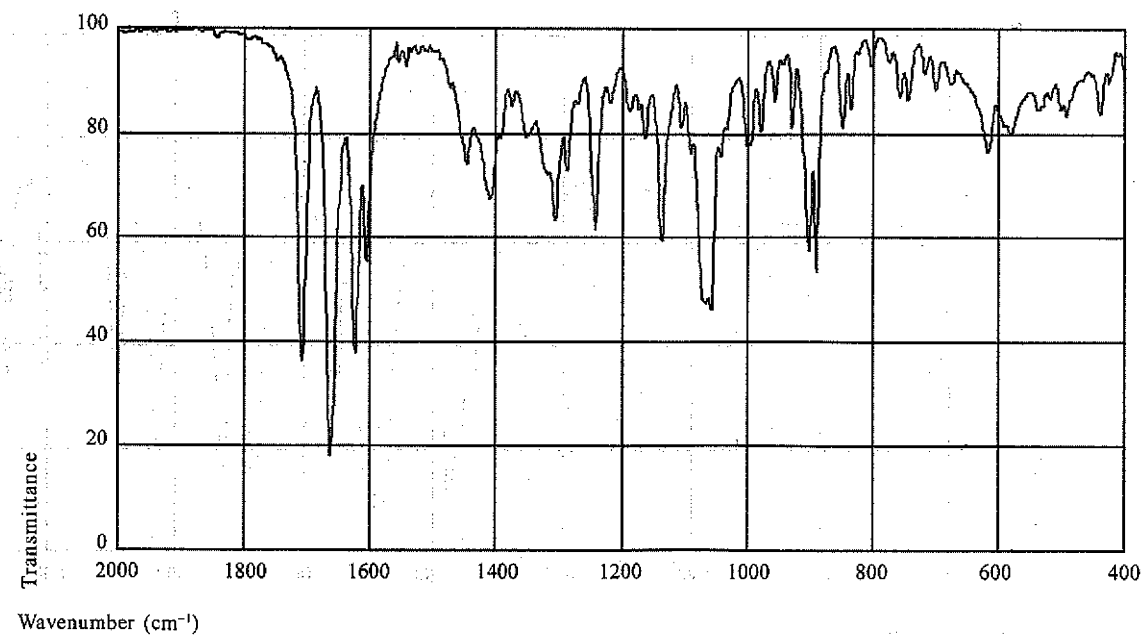
Phase: KBr disc



Trazodone Hydrochloride *FTIR* Phase: KBr disc



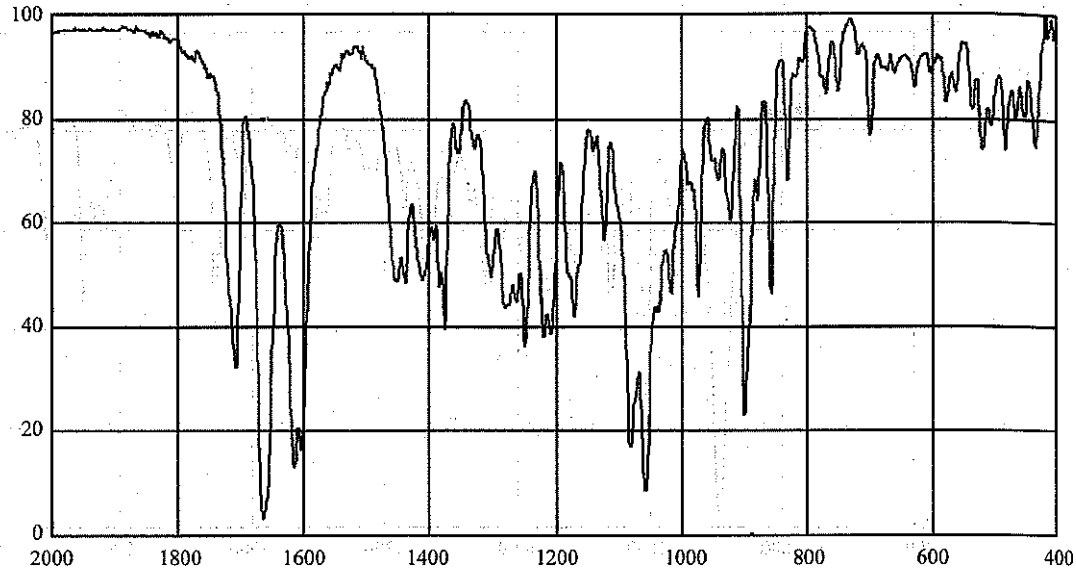
Triamcinolone *FTIR* Phase: KBr disc



Triamcinolone Acetonide

FTIR

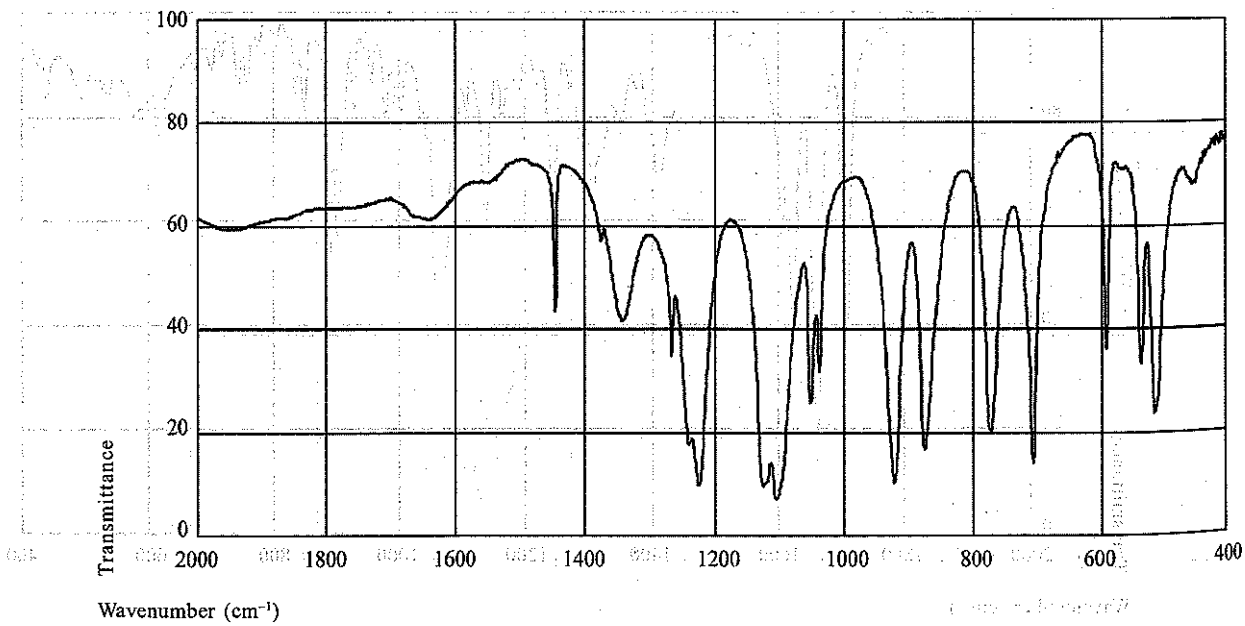
Phase: KBr disc



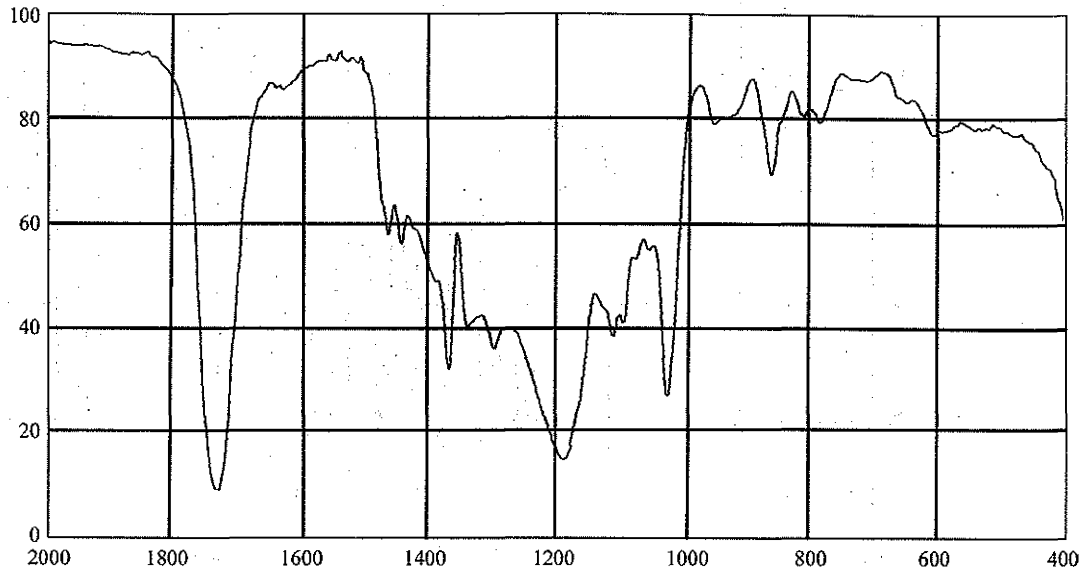
Triclofos Sodium

FTIR

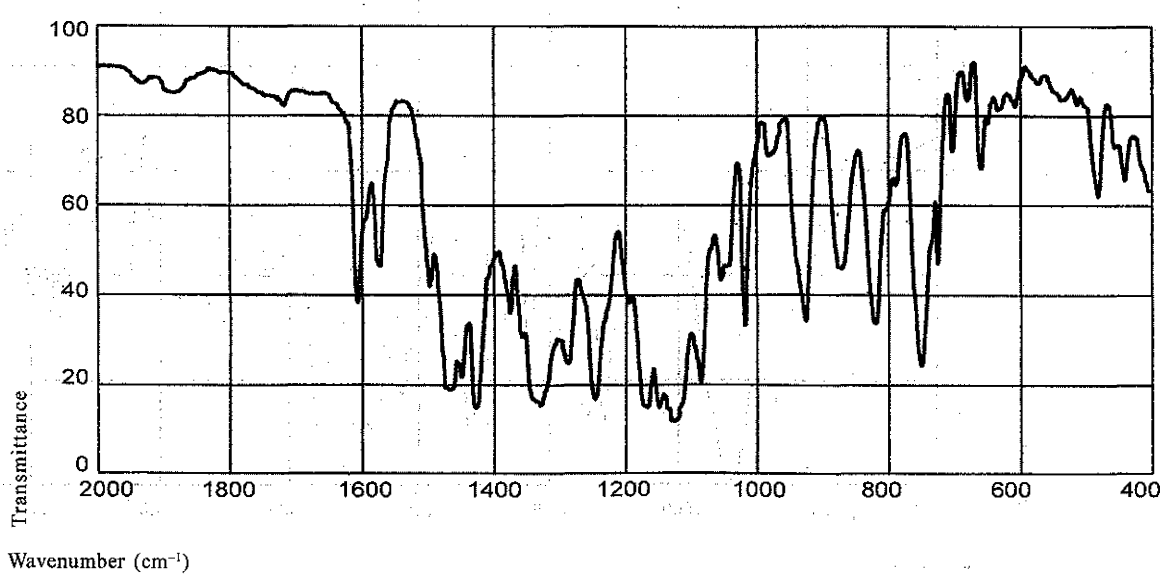
Phase: KBr disc



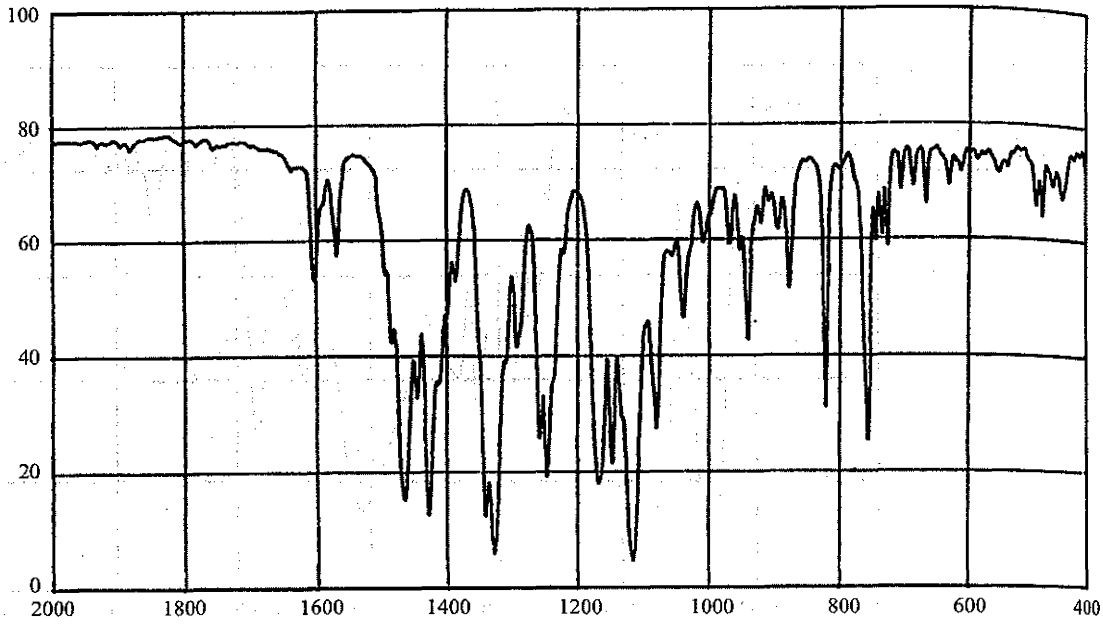
Triethyl Citrate *FTIR* Phase: KBr disc



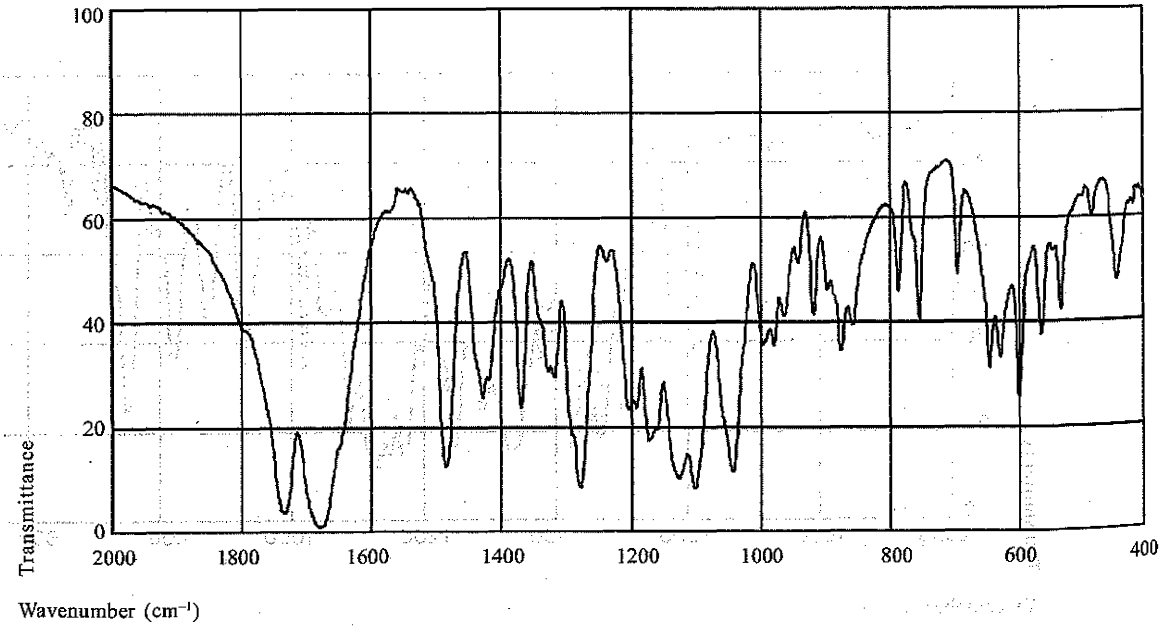
Trifluoperazine *Dispersive IR* Phase: Thin film



Triflupromazine Hydrochloride *FTIR* Phase: KBr disc



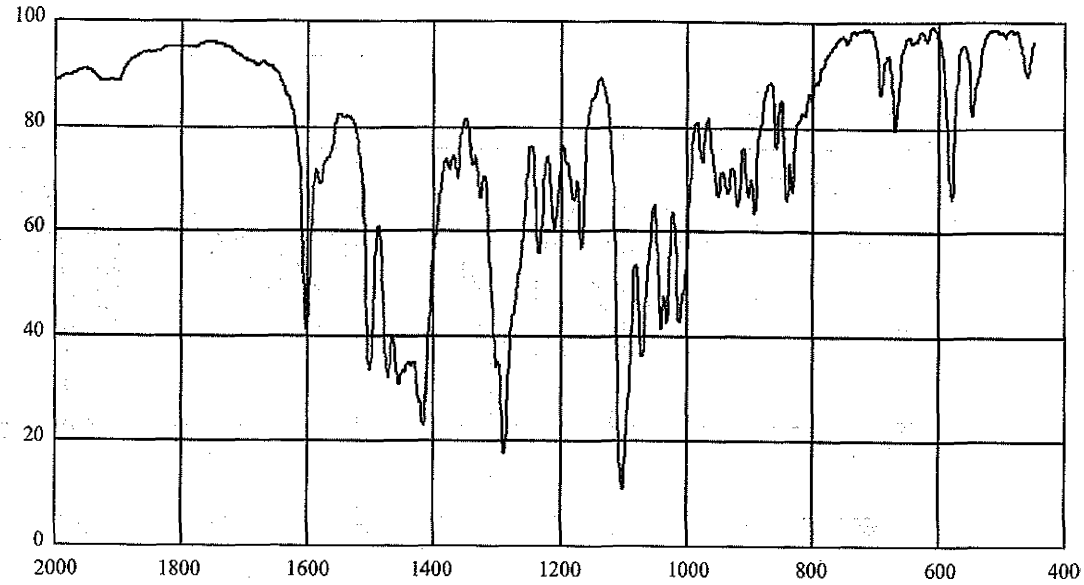
Trifluridine *FTIR* Phase: KBr disc



Trimetazidine Hydrochloride

FTIR

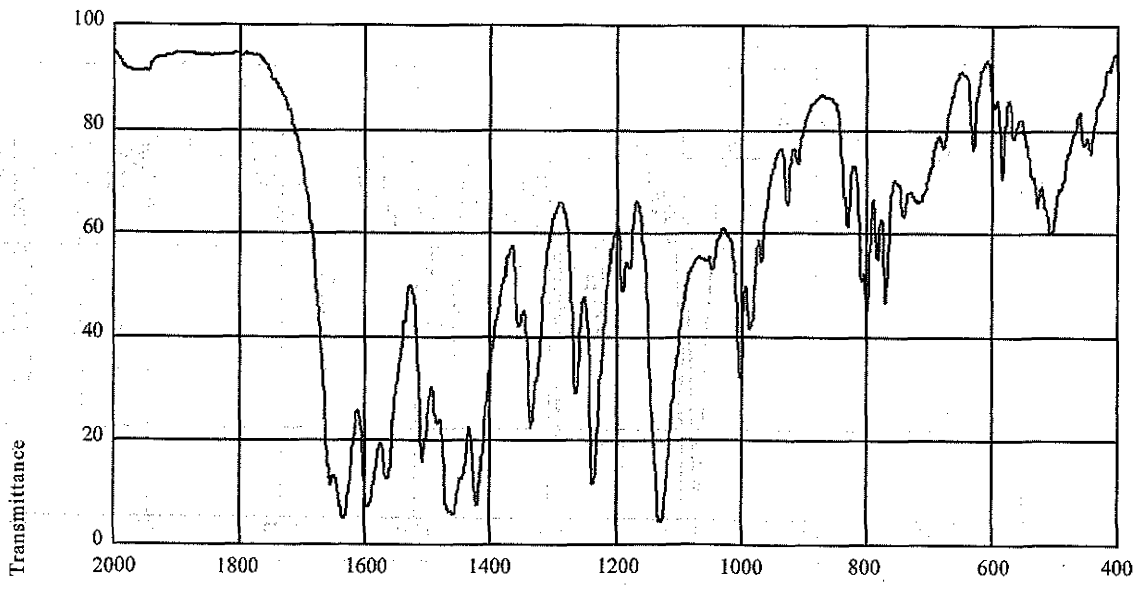
Phase: KBr disc



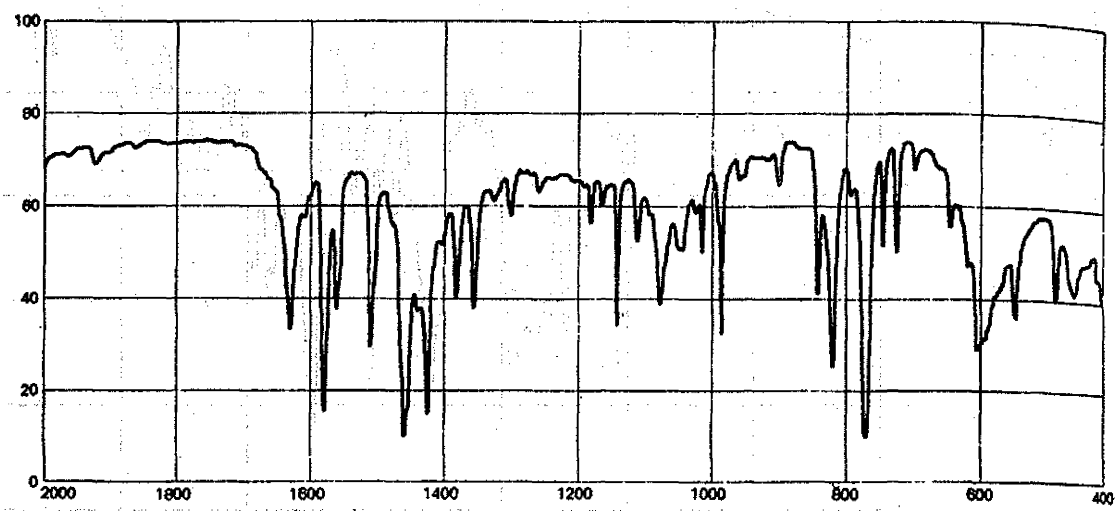
Trimethoprim

FTIR

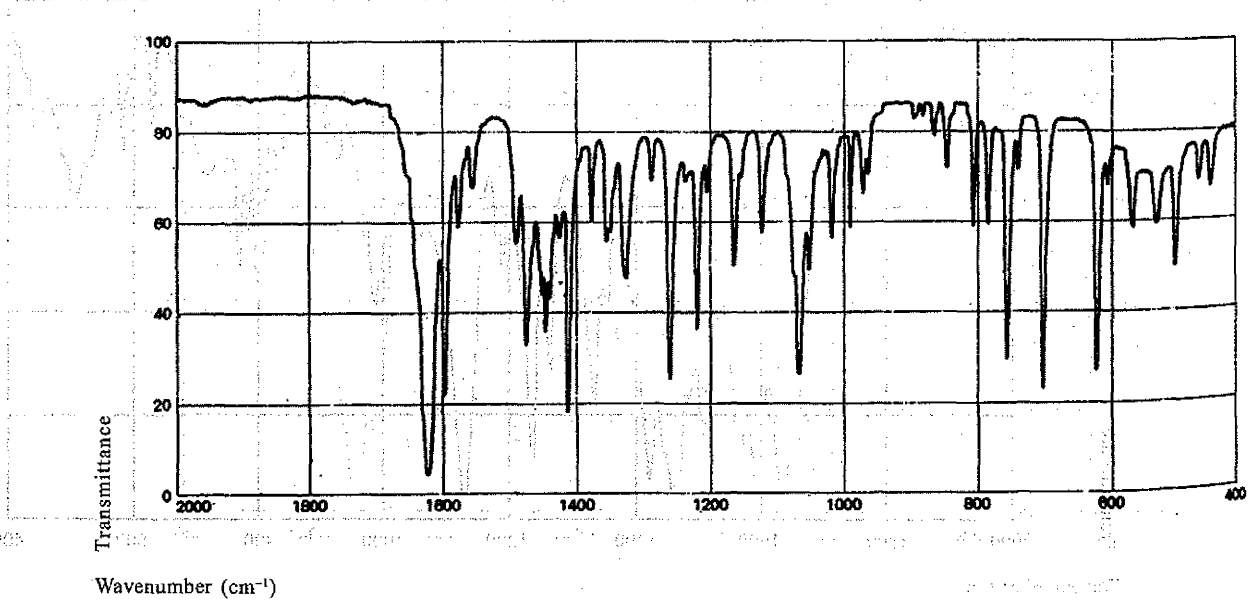
Phase: KBr disc



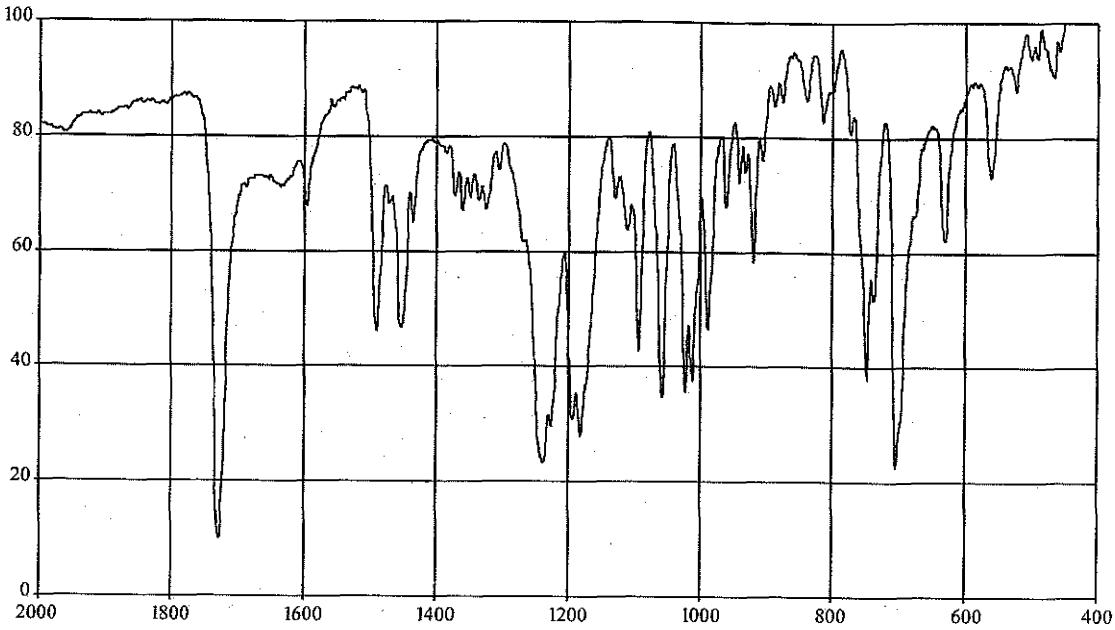
Triprolidine Hydrochloride *Dispersive IR* Phase: KCl disc



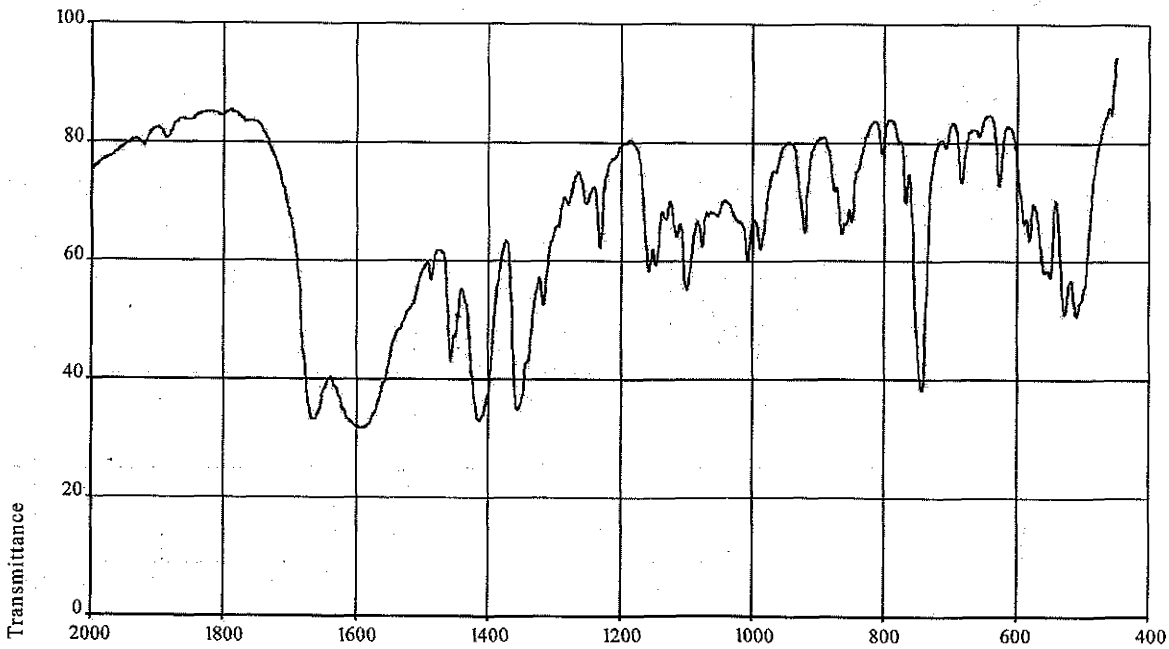
Tropicamide *Dispersive IR* Phase: KBr disc



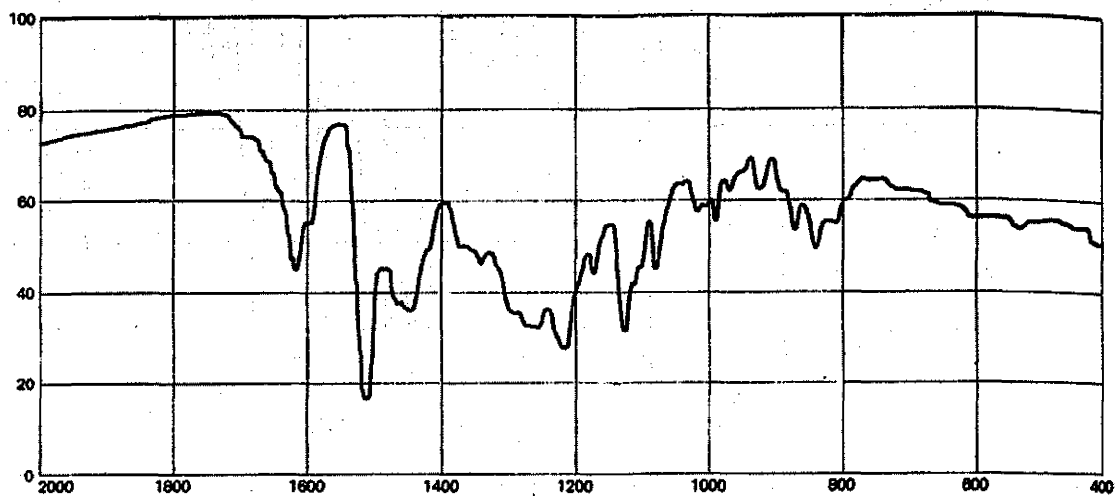
Trospium Chloride *FTIR* Phase: KBr disc



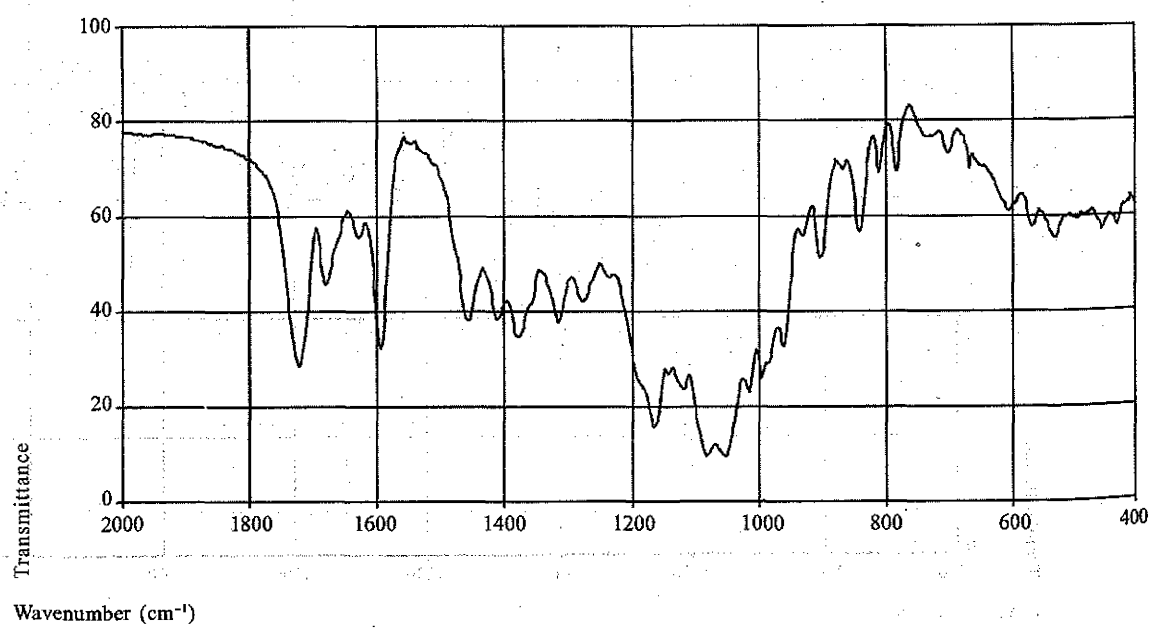
Tryptophan *FTIR* Phase: KBr disc



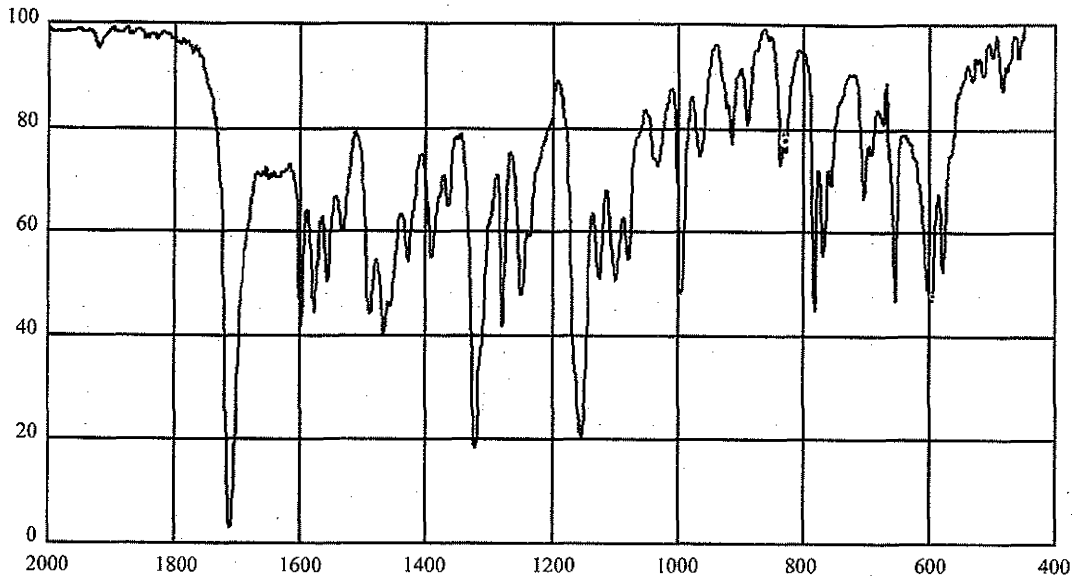
Tubocurarine Chloride *Dispersive IR* Phase: KCl disc



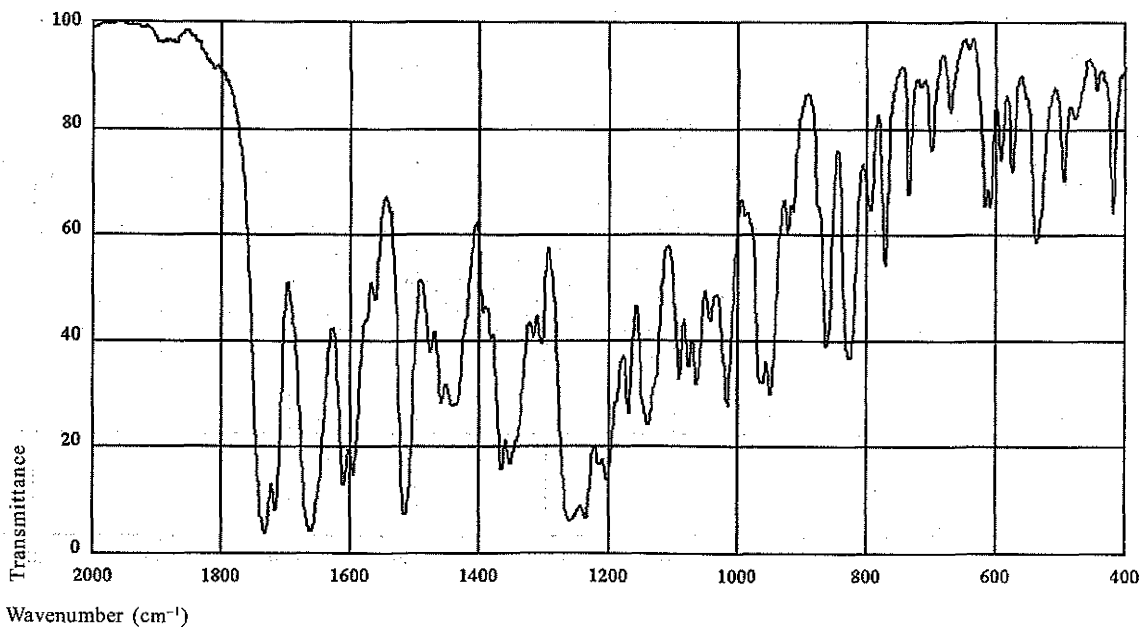
Tylosin *FTIR* Phase: KBr disc



Udenafil *FTIR* Phase: KBr disc



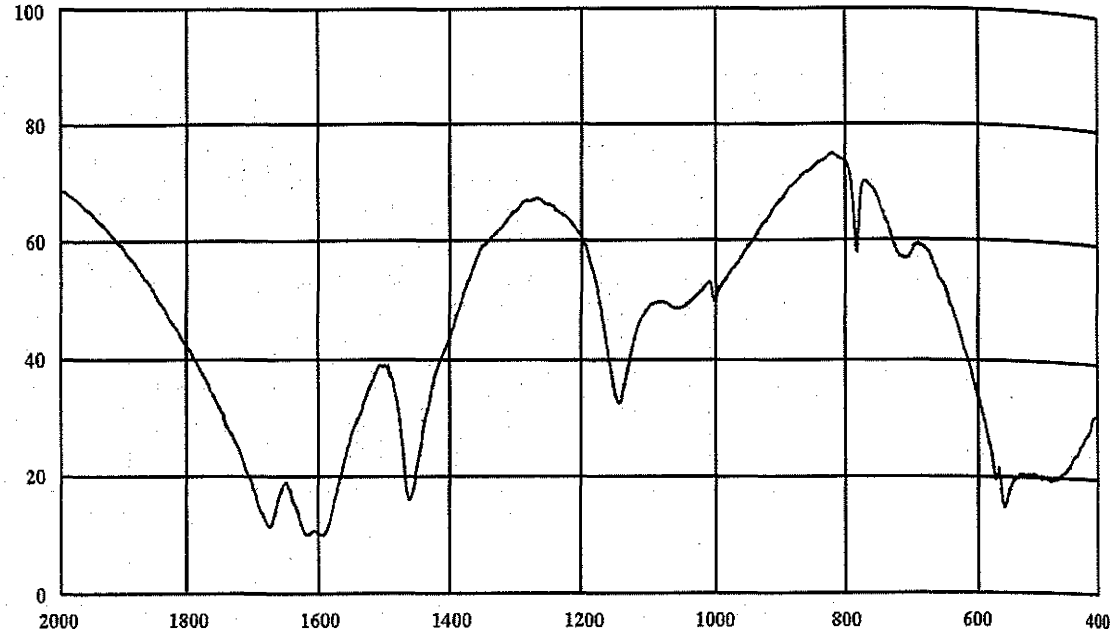
Ulipristal Acetate *FTIR* Phase: KBr disc



Urea

FTIR

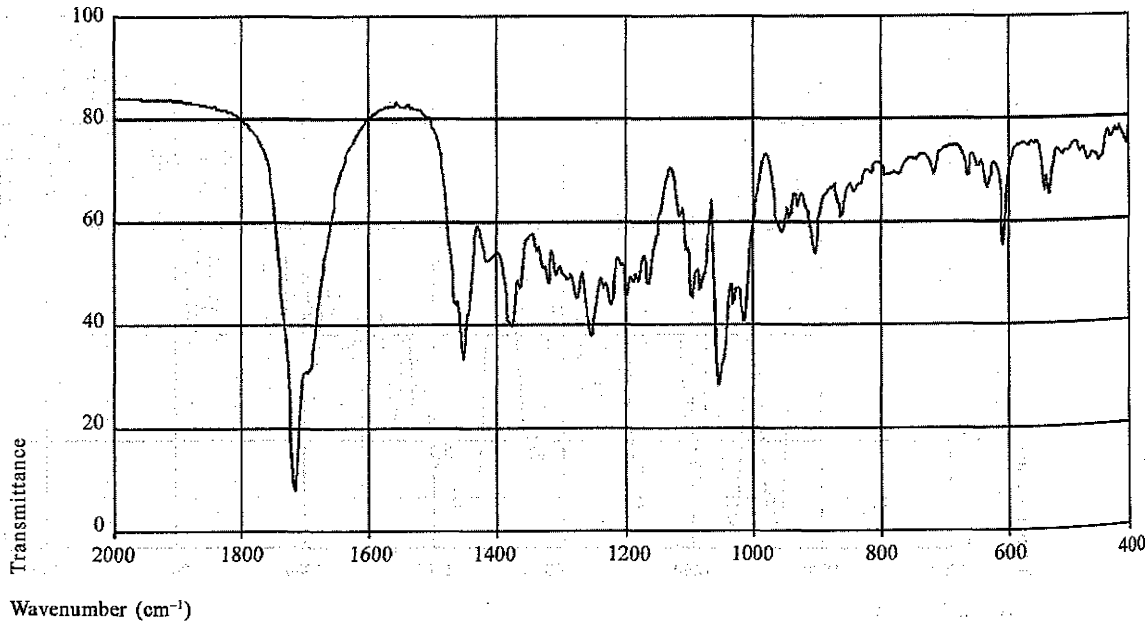
Phase: KBr disc



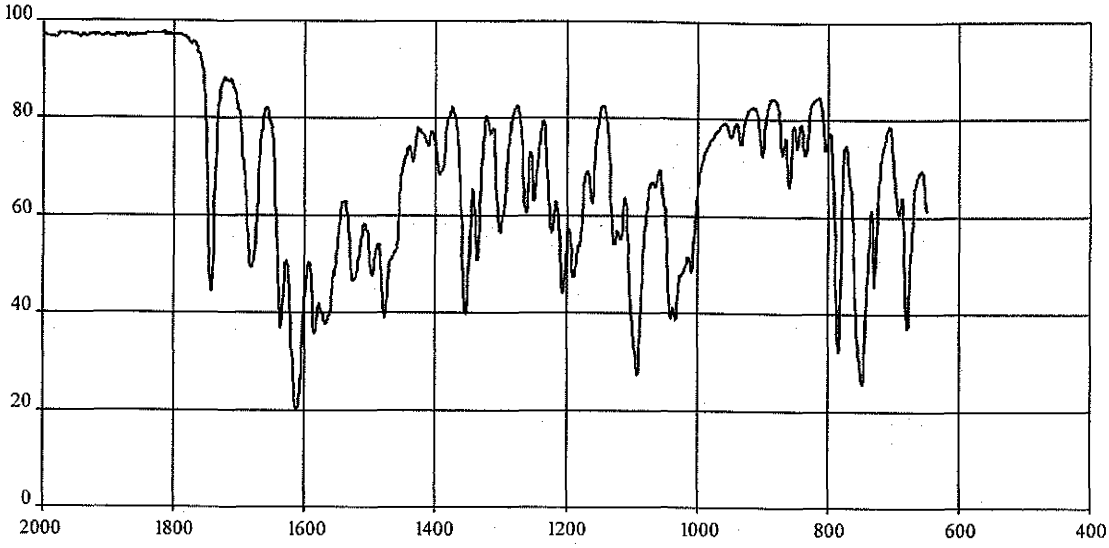
Ursodeoxycholic Acid

FTIR

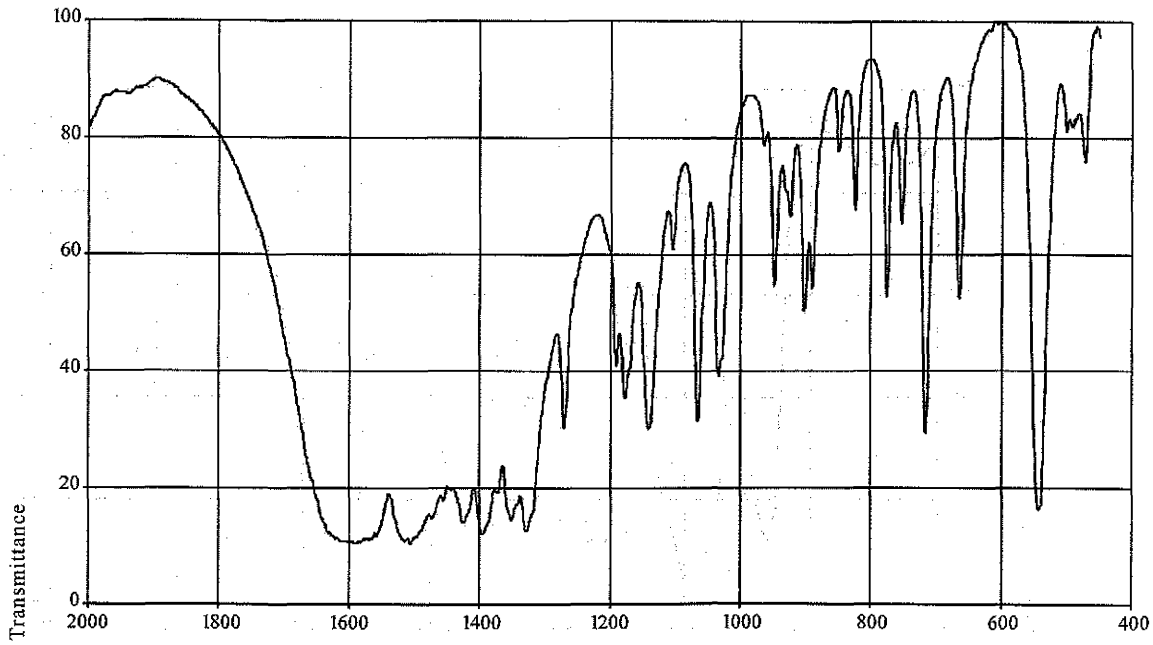
Phase: KBr disc



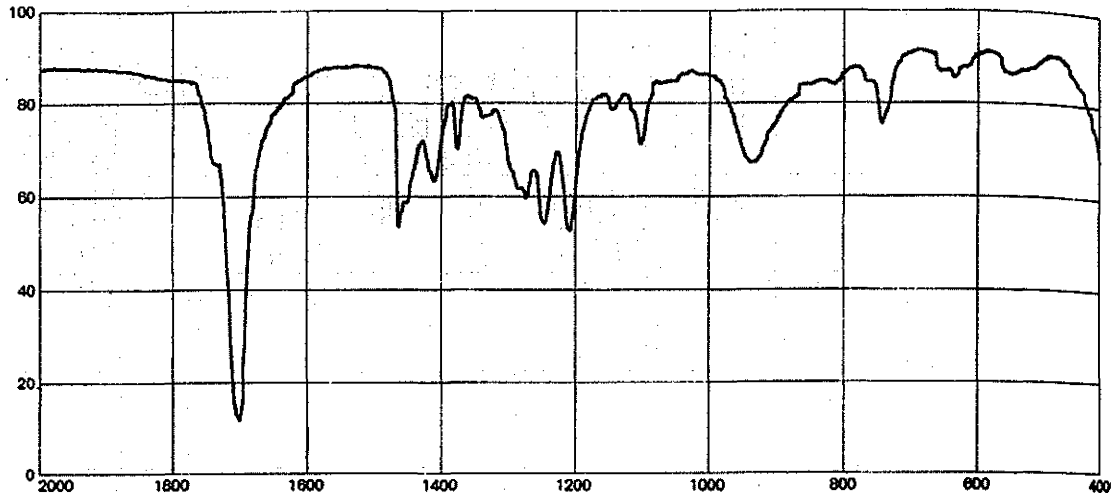
Valacyclovir Hydrochloride *FTIR* Phase: KBr disc



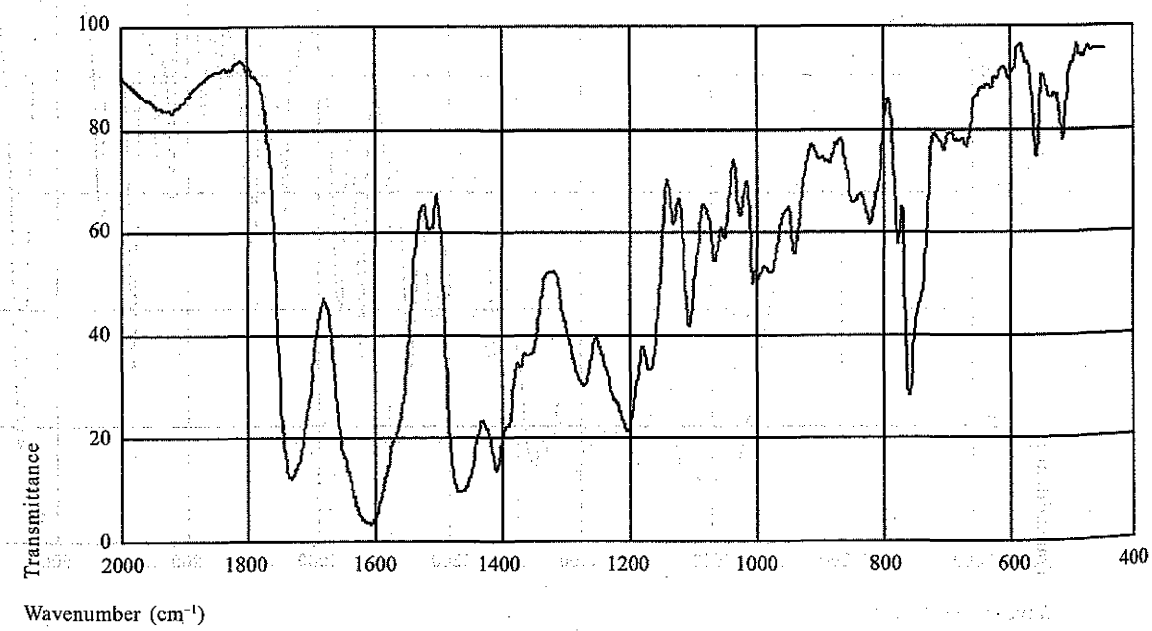
Valine *FTIR* Phase: KBr disc



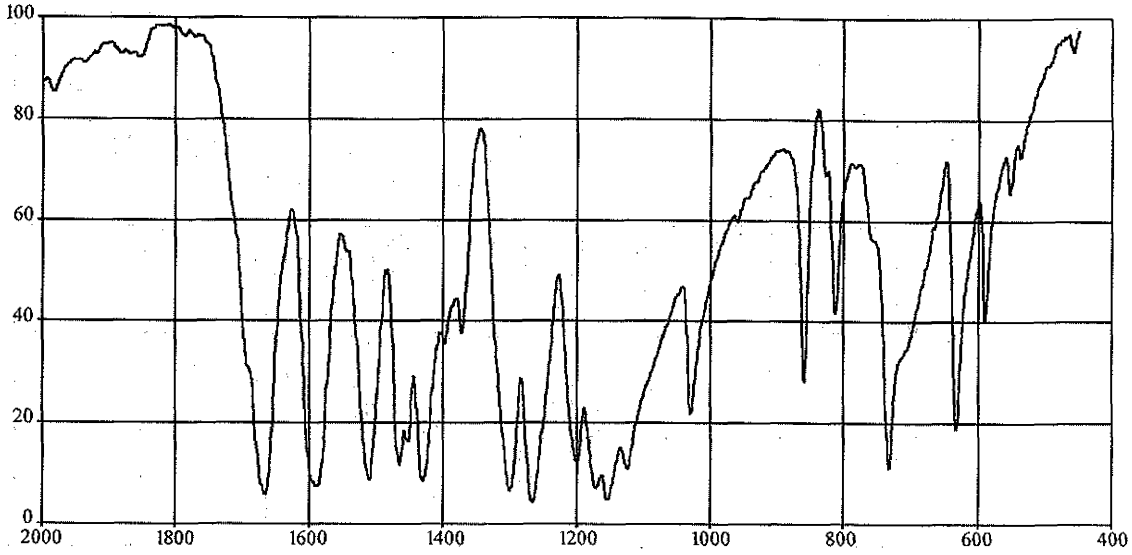
Valproic Acid *Dispersive IR* Phase: Thin film



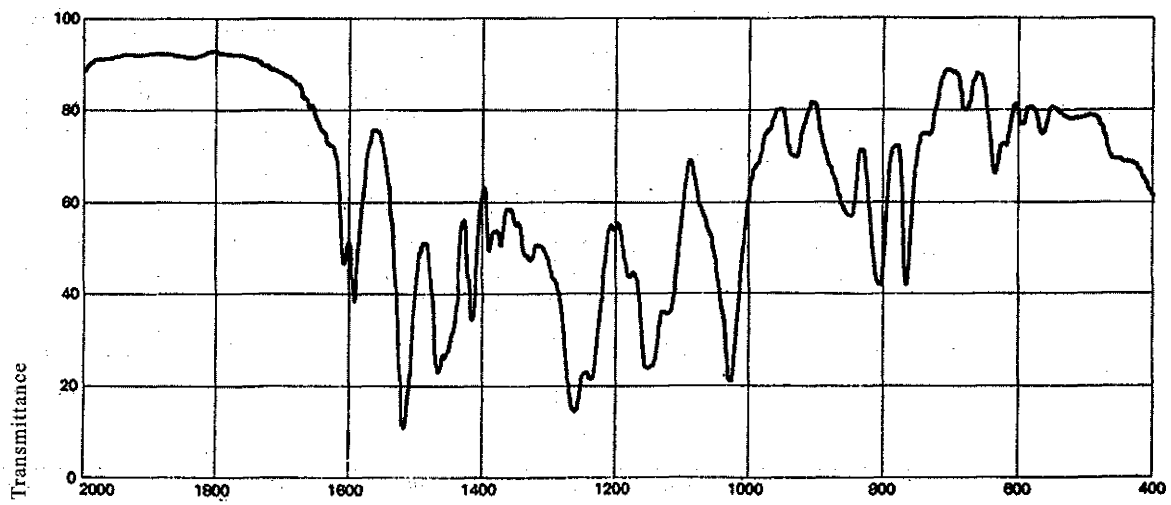
Valsartan *FTIR* Phase: KBr disc



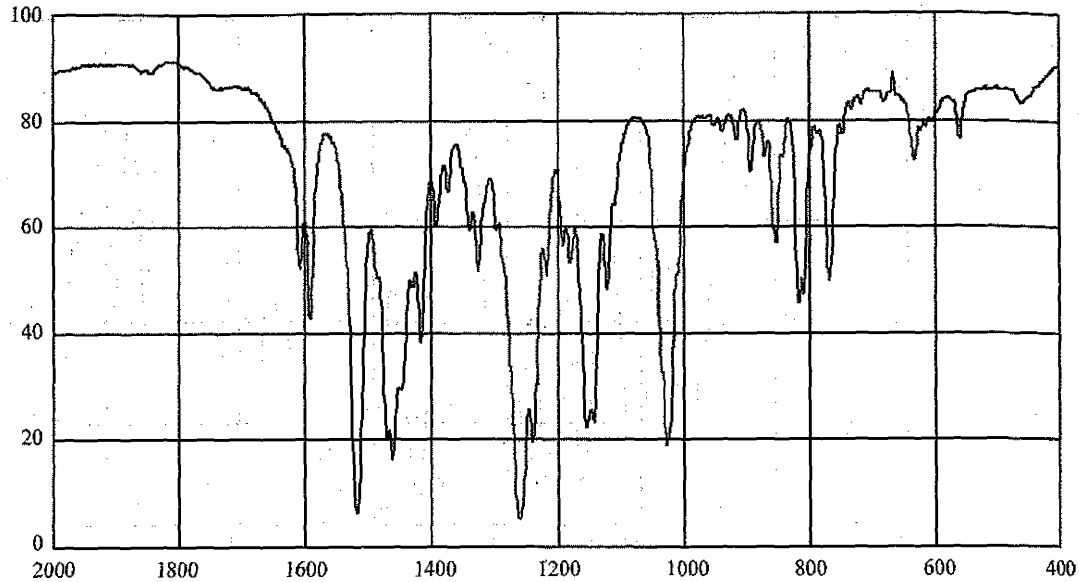
Vanillin *FTIR* Phase: KBr disc



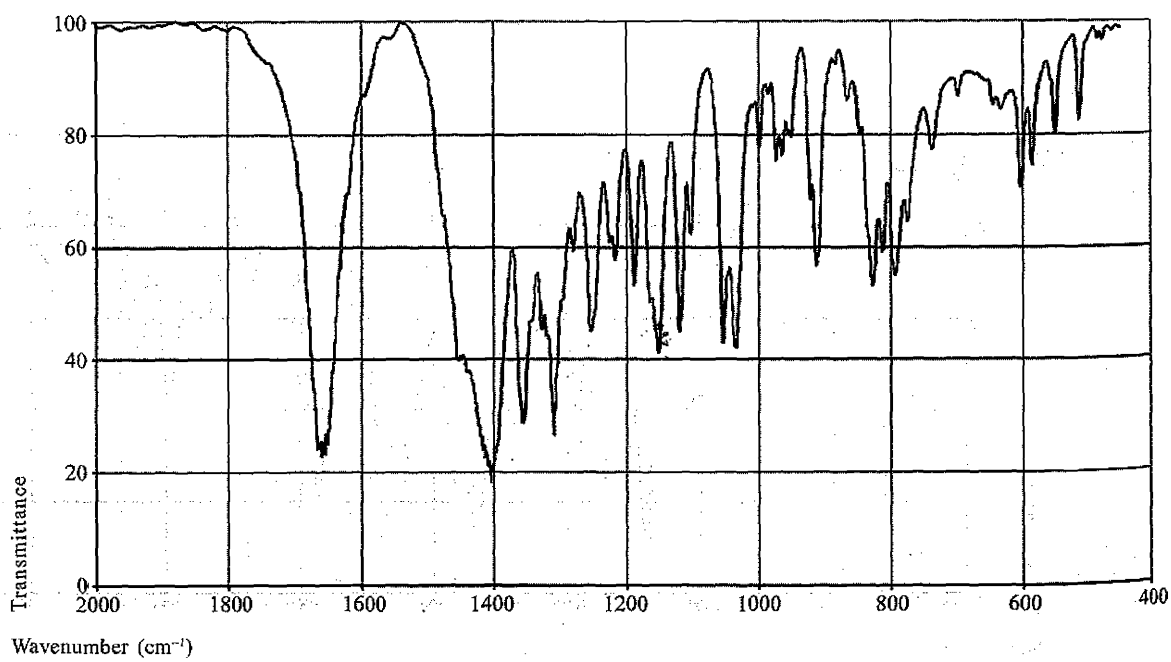
Verapamil *Dispersive IR* Phase: Thin film



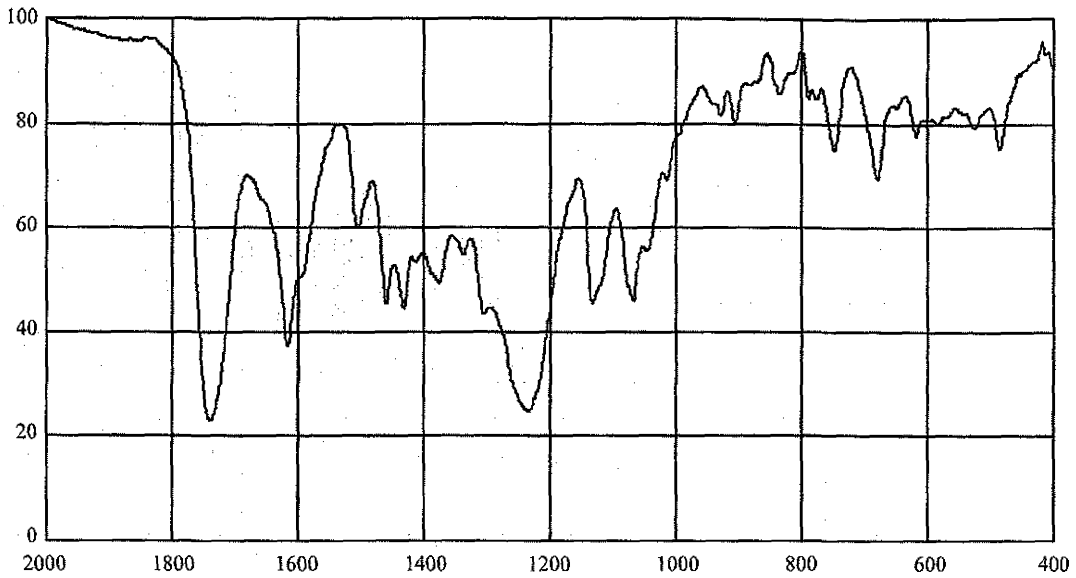
Verapamil Hydrochloride *FTIR* Phase: KCl disc



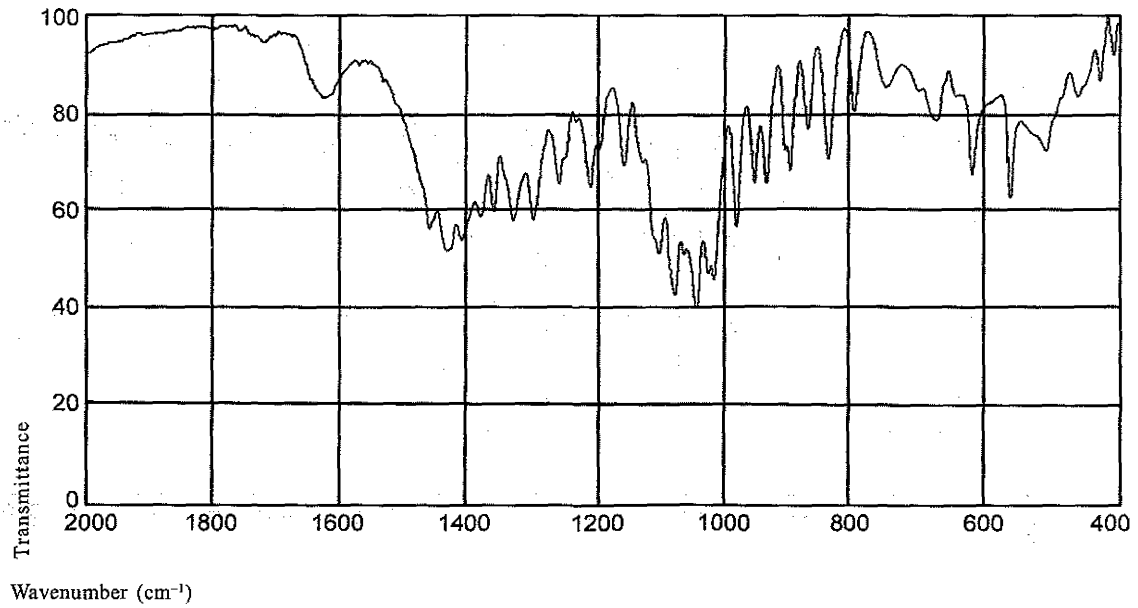
Vildagliptin *FTIR* Phase: KBr disc



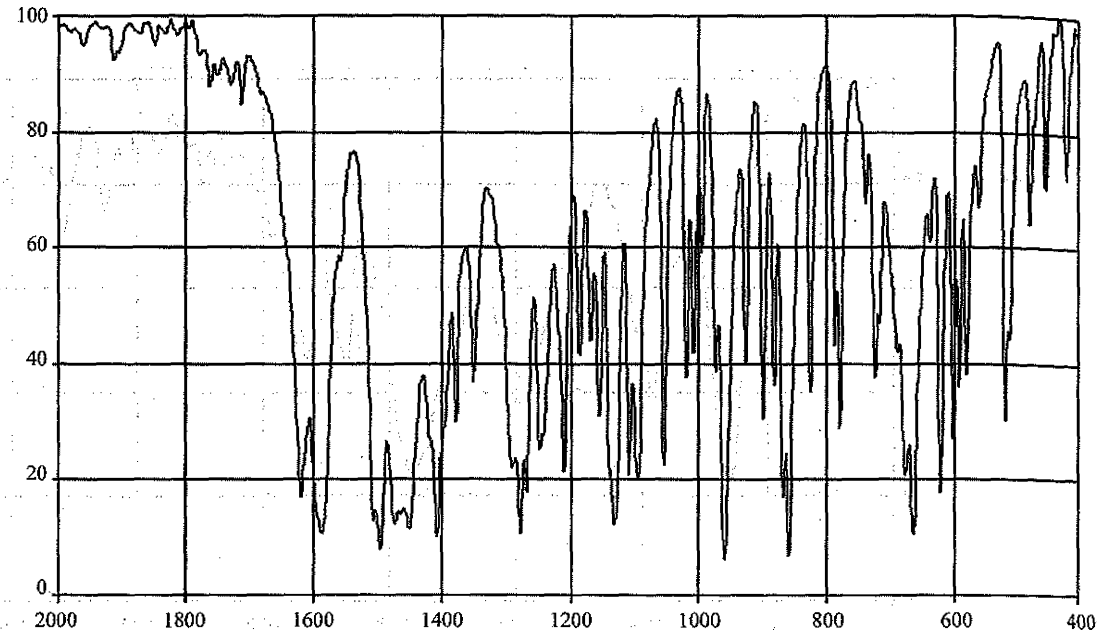
Vinorelbine *FTIR* Phase: KBr disc



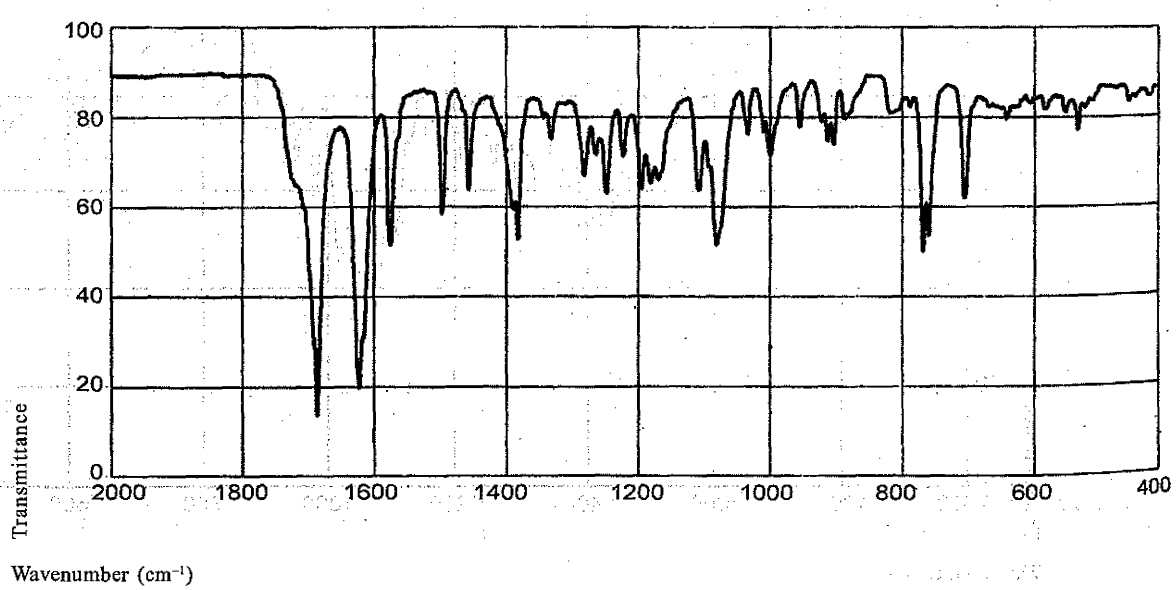
Voglibose *FTIR* Phase: KBr disc



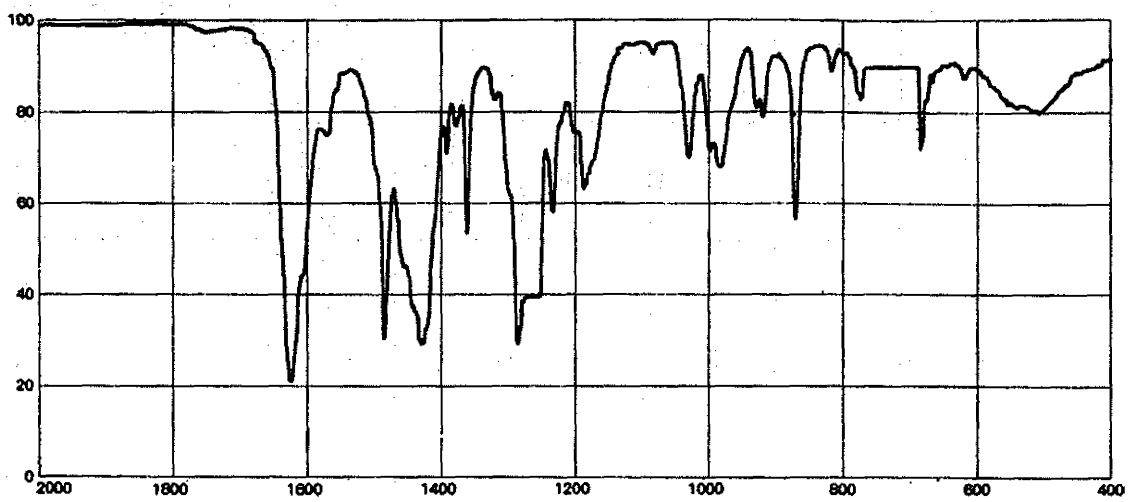
Voriconazole *FTIR* Phase: KBr disc



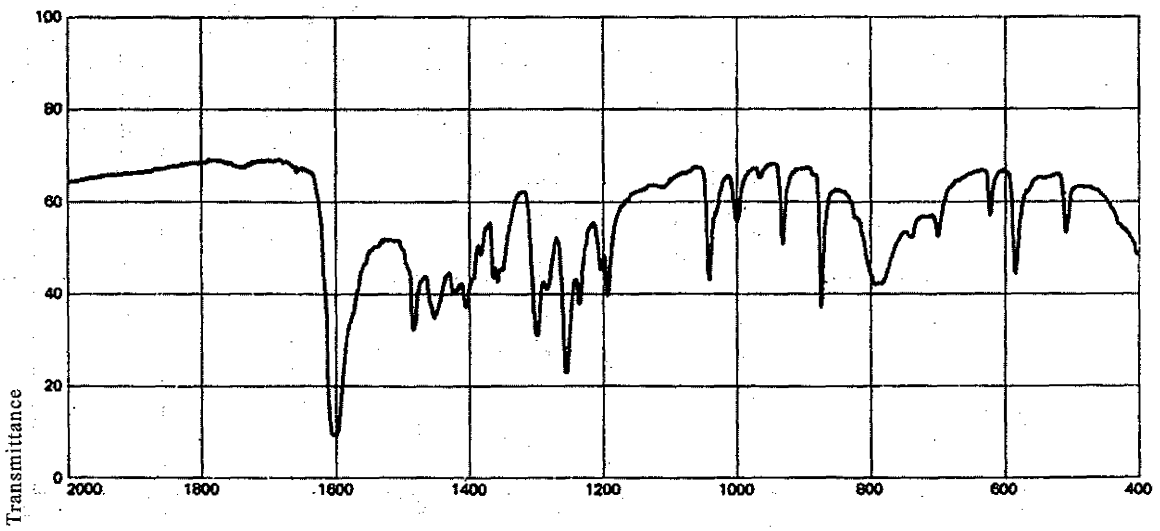
Warfarin *Dispersive IR* Phase: KBr disc



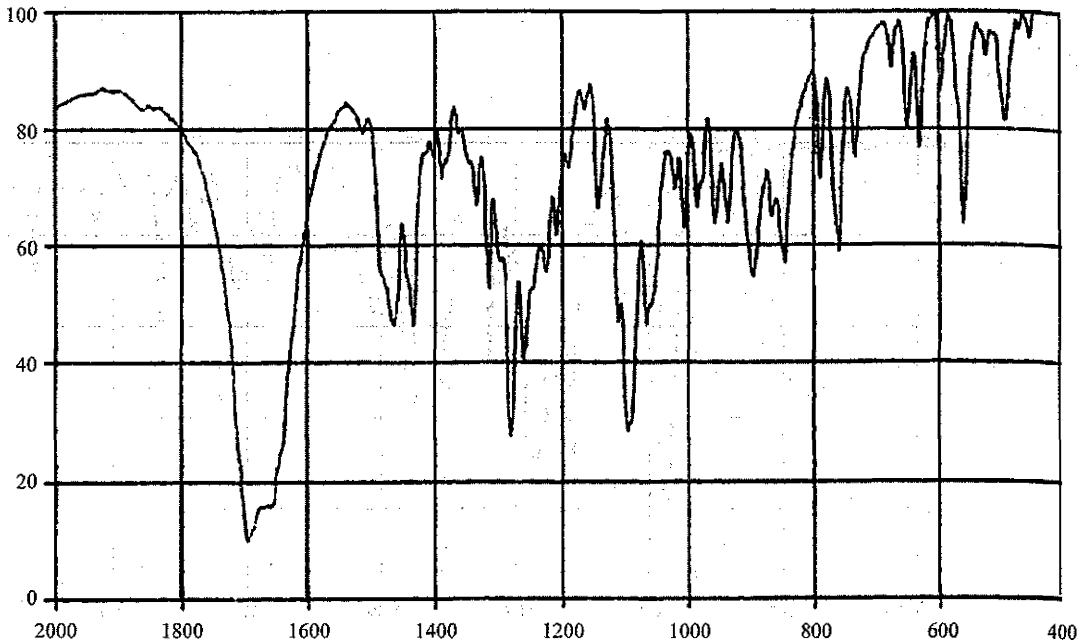
Xylometazoline *Dispersive IR* Phase: 5 per cent w/v solution in CH_2Cl_2 Thickness 0.1 mm



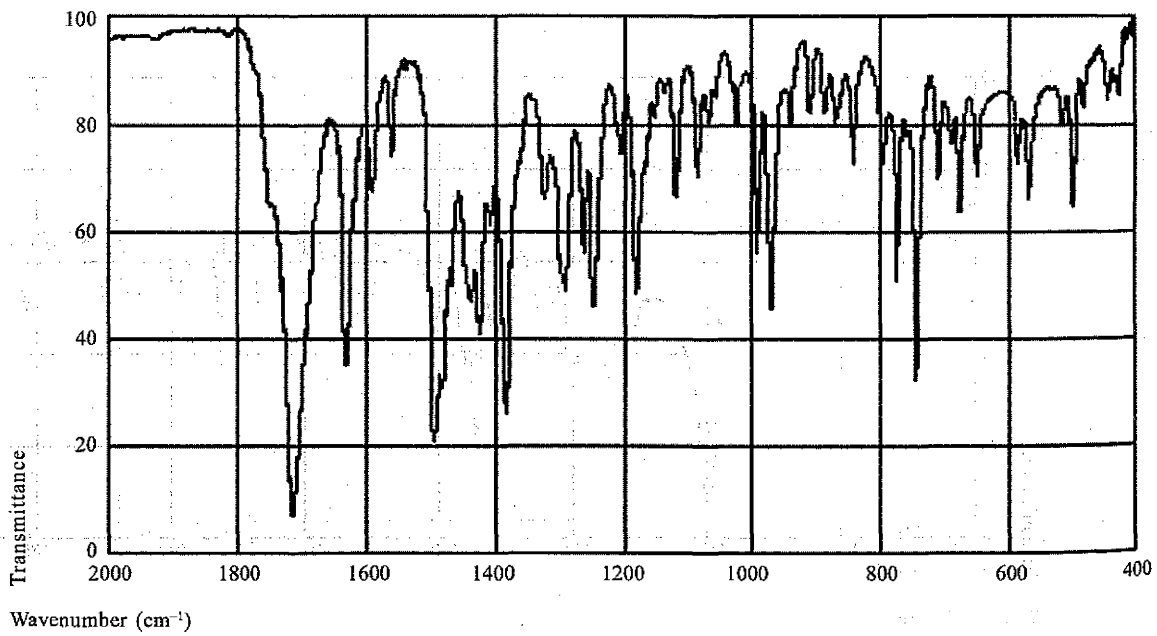
Xylometazoline Hydrochloride *Dispersive IR* Phase: KCl disc



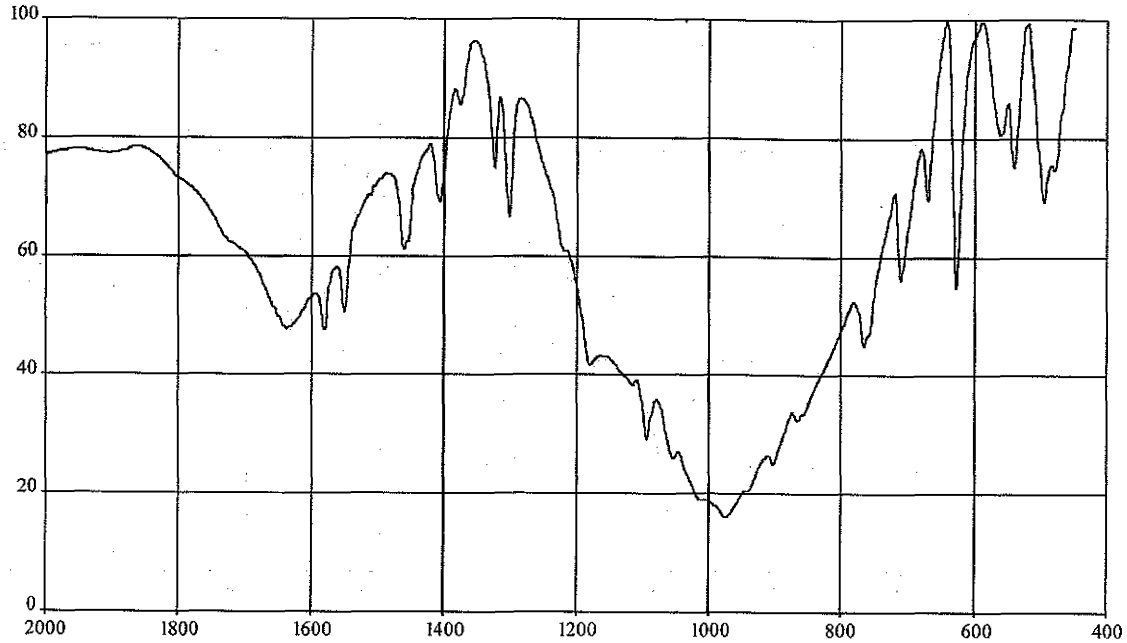
Zidovudine *FTIR* Phase: KBr disc



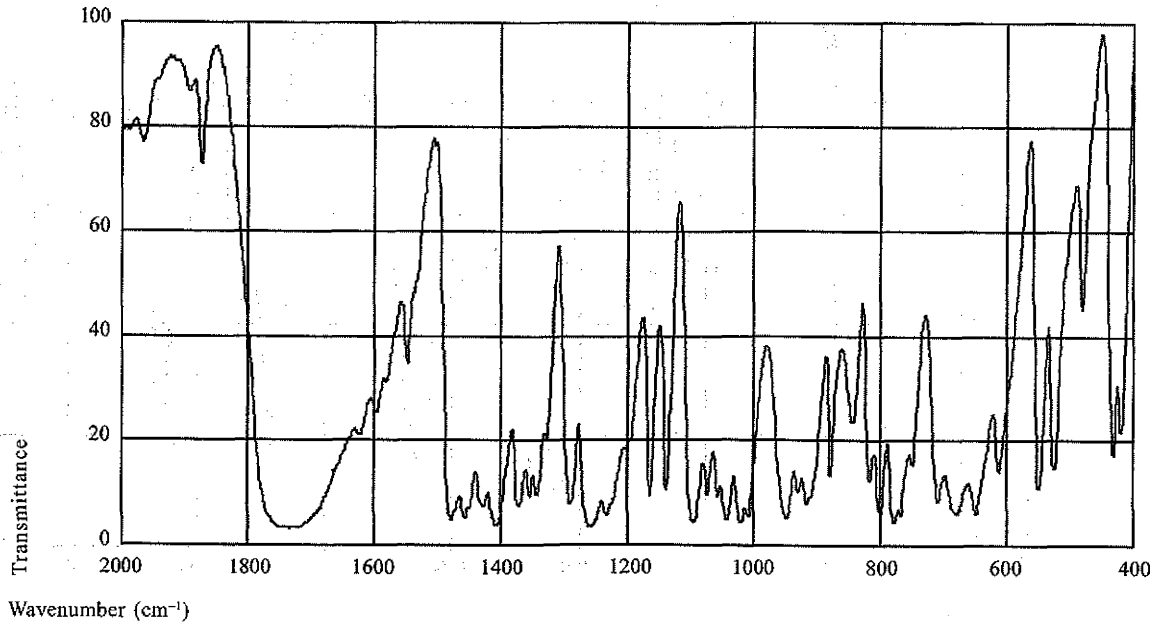
Ziprasidone Hydrochloride Monohydrate *FTIR* Phase: KBr disc



Zoledronic Acid *FTIR* Phase: KBr disc



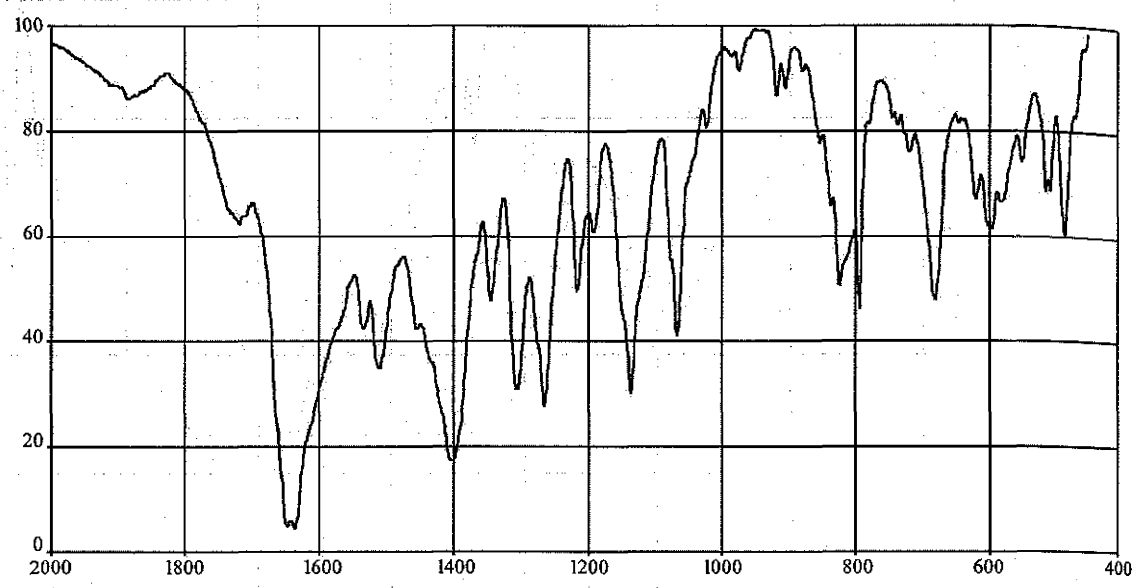
Zolmitriptan *FTIR* Phase: KBr disc



Zolpidem Tartrate

FTIR

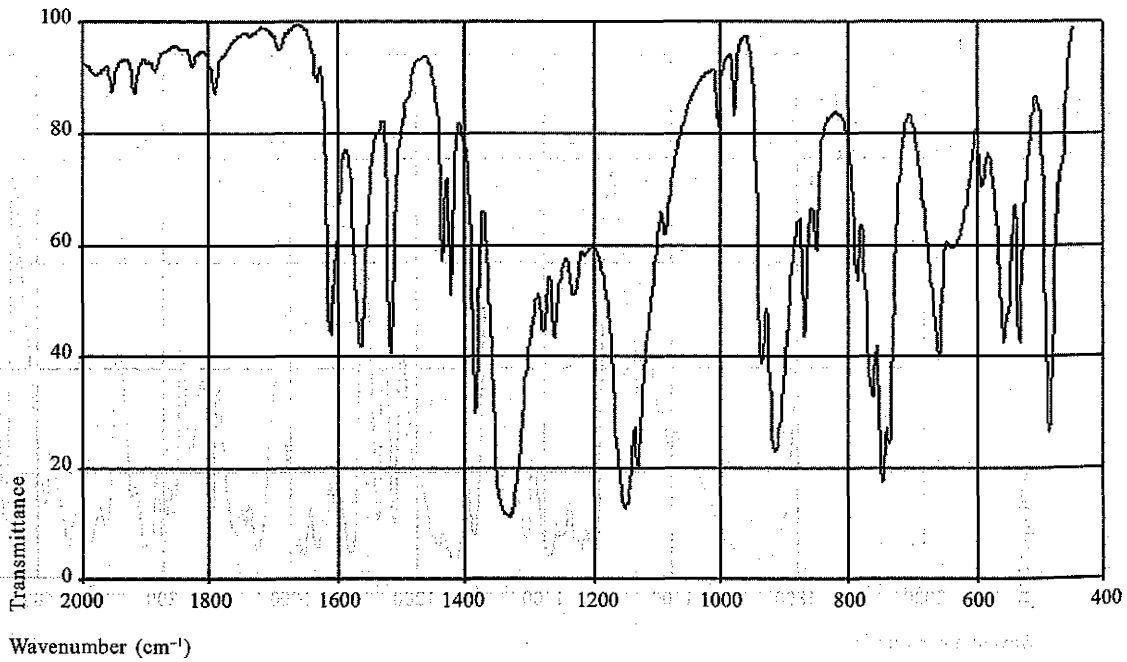
Phase: KBr disc



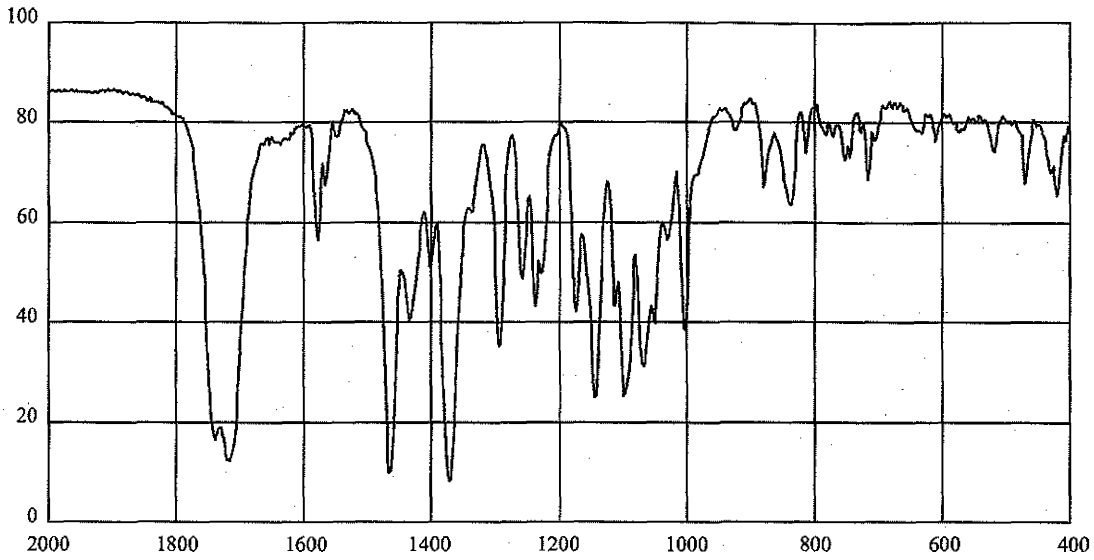
Zonisamide

FTIR

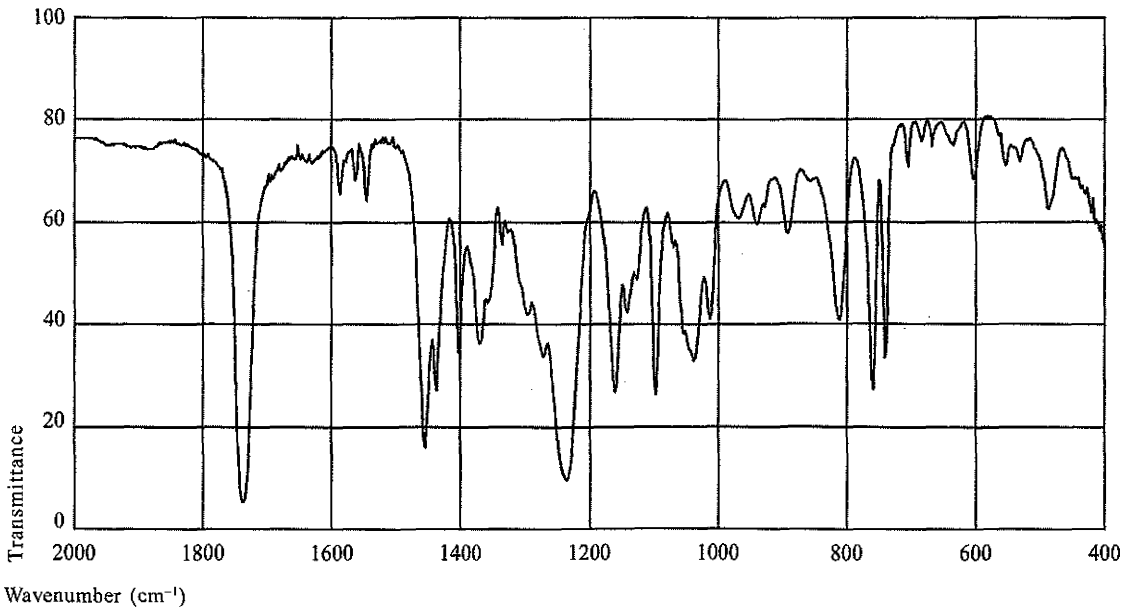
Phase: KBr disc

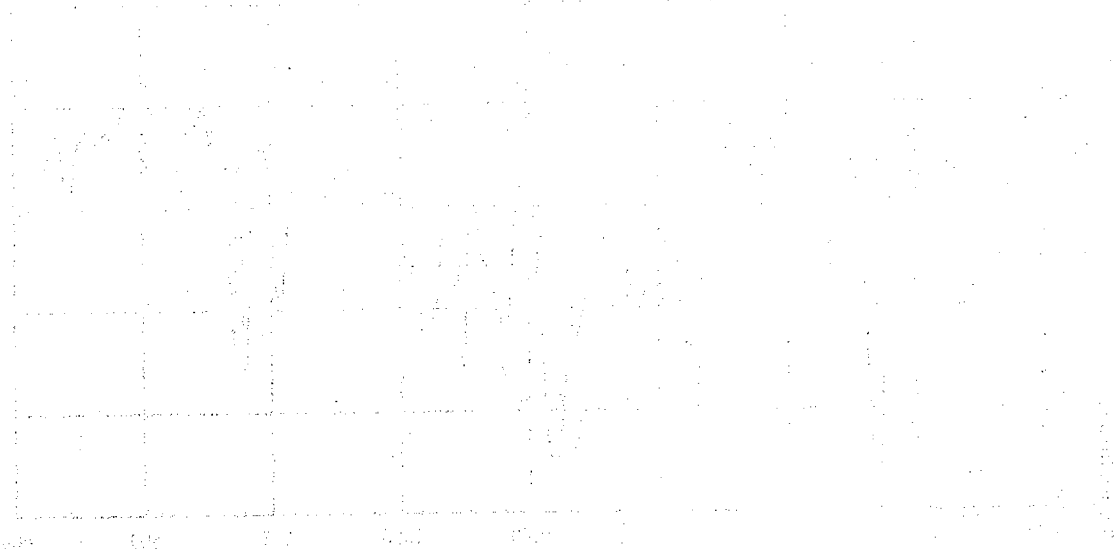


Zopiclone *FTIR* Phase: KBr disc



Zuclopenthixol Acetate *FTIR* Phase: KBr disc





3.2. Thin Layer Chromatograms of Phytopharmaceuticals, Herbs and Herbal Products

As a measure of improved objective testing for identification of crude herbs, processed herbs and herbal formulations, a test for thin-layer chromatography has been given under individual monographs wherever applicable. The test should be carried out as detailed in the individual monographs using a Indian Pharmacopopeia Botanical Reference Substance (IPBRS) for comparison. A reference substance is a standard whose botanical identity and genuineness has been well established to both genus and species level, and is issued by the Indian Pharmacopoeia Commission.

While performing the test, as a common practice, the plate shall be at least 10 cm × 5 cm or 10 cm × 10 cm in dimensions. On the plate, bands of 10 mm width shall be spotted, with the band related to substance under examination denoted as 'TEST' and that related to the reference substance denoted as 'RS'. Additional bands may be spotted from samples of the same lot or different lots. Where more samples need to be tested at a time, the size of the plate can be modified accordingly. In some cases thin-layer chromatography may also be done by applying a spot of the reference substance of the marker compound as specified in the monograph. Some phytopharmaceutical/herbal monographs may recommend the use of Indian Pharmacopoeia Phytochemical Reference Substance apart from a Indian Pharmacopoeia Botanical Reference Substance. The use of phytochemical reference standards may be omitted if the botanical reference substance is used.

As a practice, the band of the reference substance is applied at Track 1, and all other bands of the lots of the substance under examination are applied to its right as T2, T3, and so on.

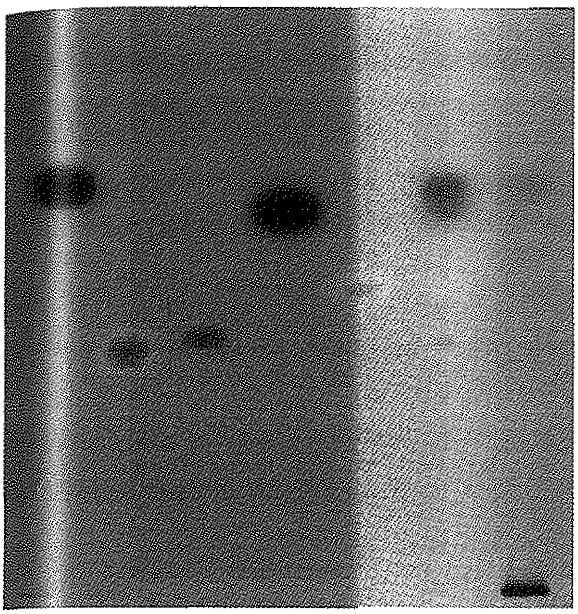
All the bands shall be applied at a height of 10 mm from the base of the plate. During development, the solvent front shall be allowed to move to at least 8 cm from the base of the plate.

Photo-documentation of the plate developed as above, for various monographs after visualization either under ultraviolet light or by any derivatisation or spraying reagents, as prescribed in the individual monographs has been reproduced here. Examination of the plates at the end of development shall be done as a matter of routine by first visualizing under ultraviolet light at 254 nm and 366 nm in a UV viewing cabinet followed by any derivatization or spraying reagents, as prescribed in the individual monographs. The profiles may be examined for similarities with the reference substances. These have been provided by the individual participating organizations that assisted in the development of monographs and methods have undergone collaborative testing.

Reproduced below are the typical TLC profiles of 3.2.1. Phytopharmaceuticals, 3.2.2. Herbs and herbal products for which a monograph appears in this edition of IP. These profiles have been provided for guidance purposes only. Comparison to same may be adopted routinely, though not for regulatory purposes as they do not form part of the monograph.

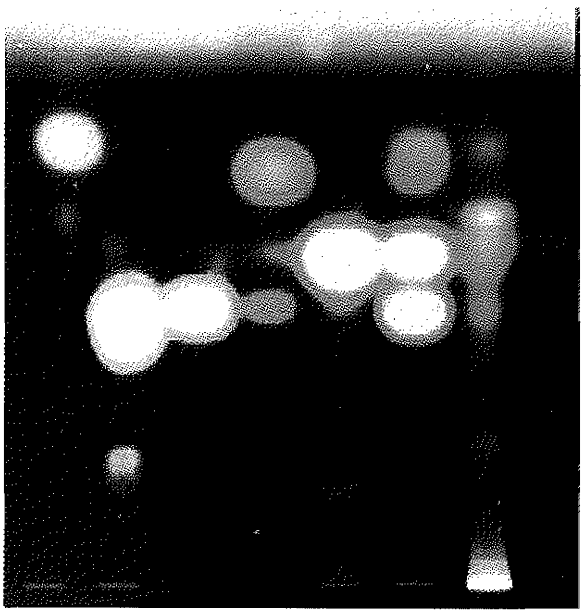
UNAUTHORISED COPY

Aegle marmelos PPI



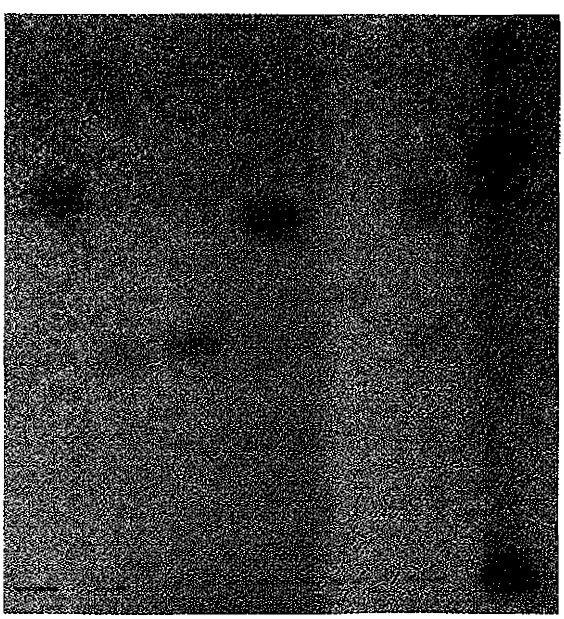
IPRS1 IPRS2 IPRS3 IPRS4 IPRS5 IPRSMIX TEST

(Under UV light at 254 nm)



IPRS1 IPRS2 IPRS3 IPRS4 IPRS5 IPRSMIX TEST

(Under UV light at 366 nm)



IPRS1 IPRS2 IPRS3 IPRS4 IPRS5 IPRSMIX TEST

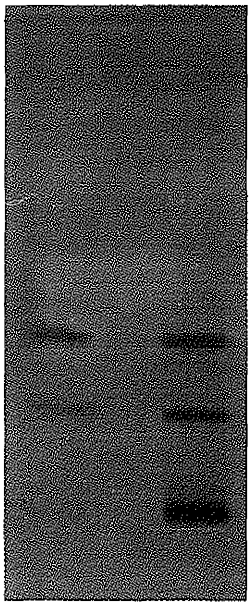
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

(IPRS1 = Marmelosin IPRS, IPRS2 = Scopoletin IPRS, IPRS3 = Marmesin IPRS, IPRS4 = Psoralen IPRS and IPRS5 = Umbelliferone IPRS)

TLC Chromatogram of Aegle marmelos PPI

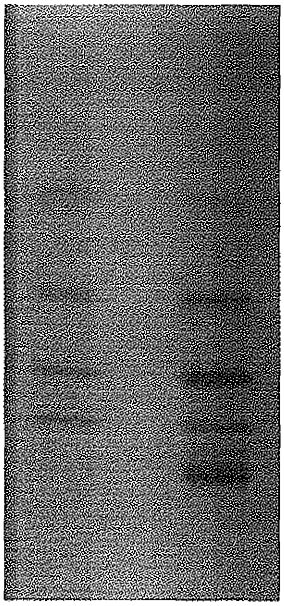
UNAUTHORISED COPY

Andrographis paniculata PPI



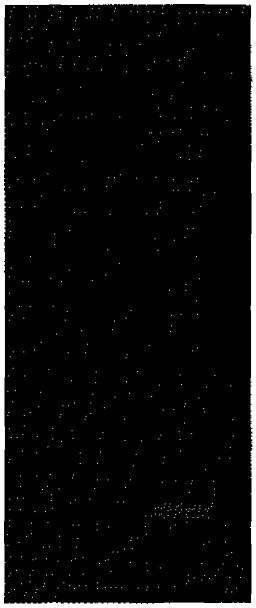
IPRSMIX TEST

(Under UV light at 254 nm)



IPRSMIX TEST

(Under UV light at 366 nm)

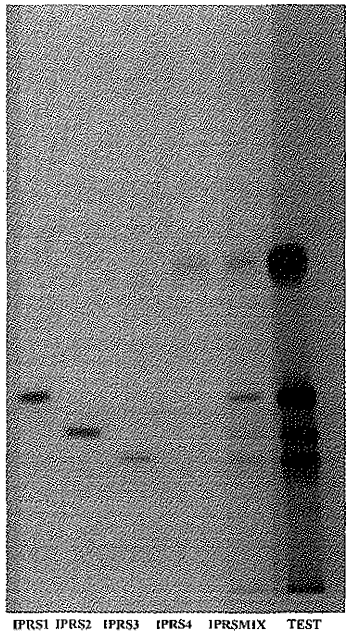


IPRSMIX TEST

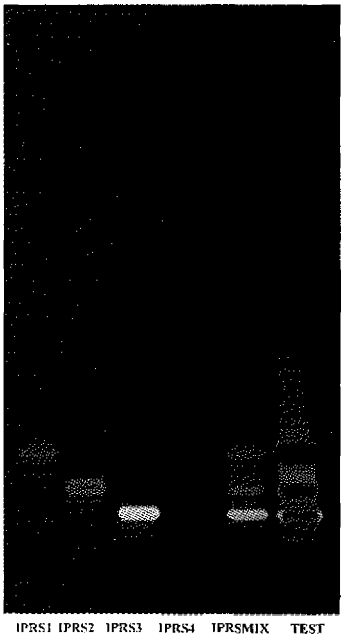
(Under day light after spraying with vanillin sulphuric acid reagent)

TLC Chromatogram of Andrographis paniculata PPI

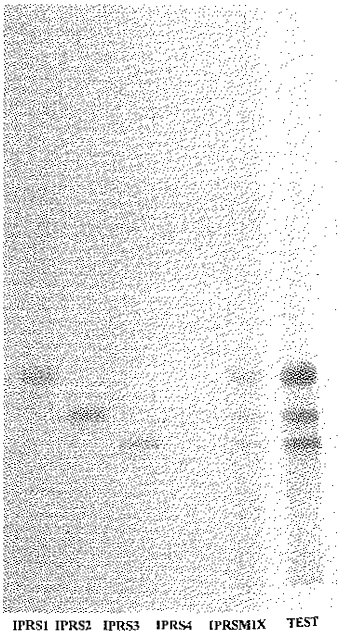
Curcuma longa PPI



(Under UV light at 254 nm)



(Under UV light at 366 nm)



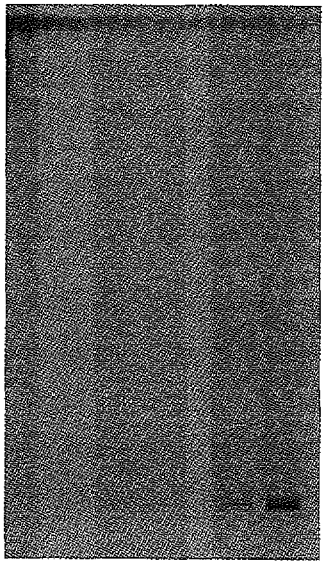
(Under day light)

(IPRS1 = Curcumin IPRS, IPRS2 = Demethoxycurcumin IPRS, IPRS3 = Bis-demethoxycurcumin IPRS and IPRS4 = ar-turmerone IPRS)

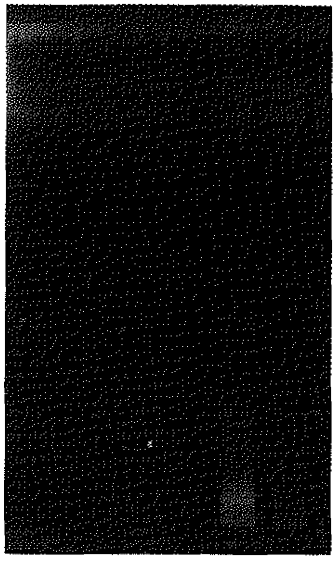
TLC Chromatogram of Curcuma longa PPI

UNAUTHORISED COPY

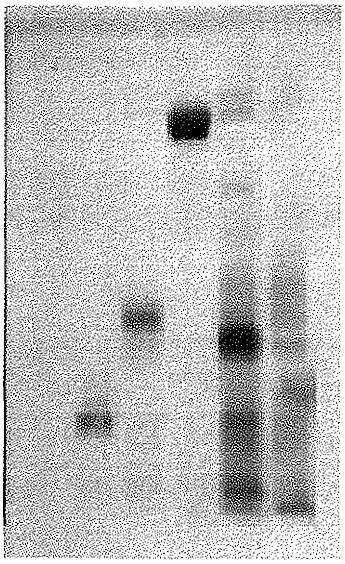
Gymnema sylvestre PPI



IPRS1 IPRS2 IPRS3 IPRS4 TEST
(Under UV light at 254 nm)



IPRS1 IPRS2 IPRS3 IPRS4 TEST
(Under UV light at 366 nm)

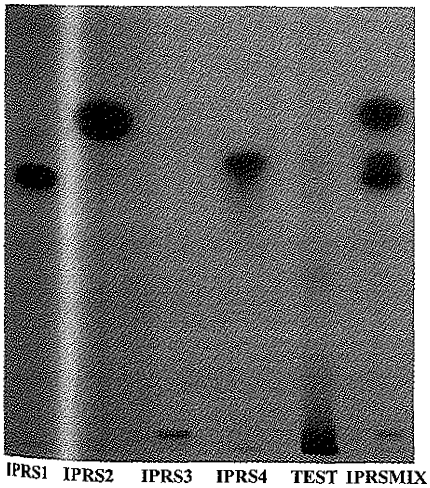


IPRS1 IPRS2 IPRS3 IPRS4 TEST
(Under day light after spraying with dilute sulphuric acid reagent)

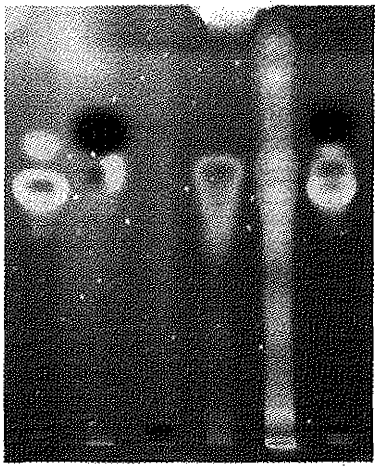
(IPRS1 = Deacylgymnemic acid IPRS, IPRS2 = Gymnemic acid IV IPRS, IPRS3 = Gymnemagenin IPRS and IPRS4 = Gymnemic acid XVII IPRS)

TLC Chromatogram of Gymnema sylvestre PPI

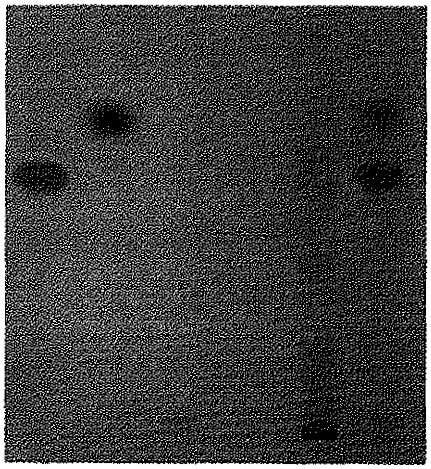
Lawsonia inermis PPI



IPRS1 IPRS2 IPRS3 IPRS4 TEST IPRSMIX
(Under UV light at 254 nm)



IPRS1 IPRS2 IPRS3 IPRS4 TEST IPRSMIX
(Under UV light at 366 nm)



IPRS1 IPRS2 IPRS3 IPRS4 TEST IPRSMIX
(Under white light)

(IPRS1 = Quercetin IPRS, IPRS2 = Lawsone IPRS, IPRS3 = Luteolin-7-O-glucoside IPRS and IPRS4 = Apigenin IPRS)

TLC Chromatogram of Lawsonia inermis PPI

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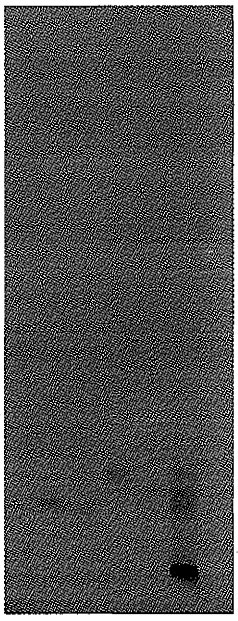
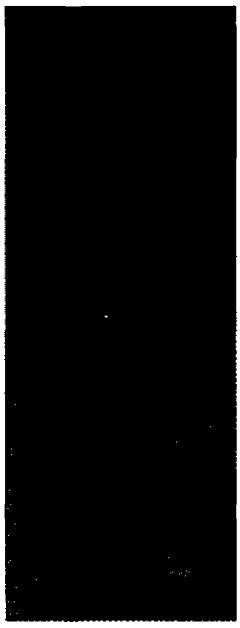
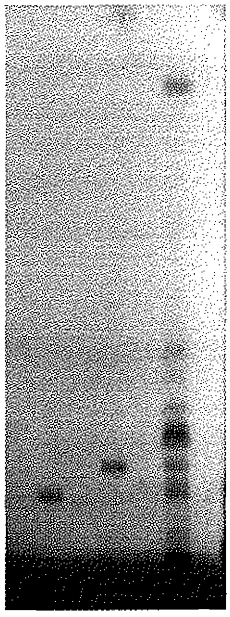
Phyllanthus amarus PPI



(Under UV light at 254 nm)



(Under UV light at 366 nm)

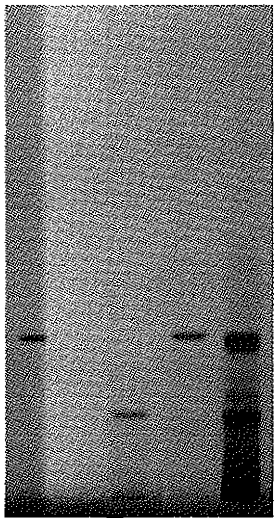


(Under day light after spraying with vanillin sulphuric acid reagent)

(IPRS1 = Phyllanthin IPRS, IPRS2 = Hypophyllanthin IPRS, IPRS3 = Nirtetralin IPRS and IPRS4 = Niranthin IPRS)

TLC Chromatogram of Phyllanthus amarus PPI

Silybum marianum Dry Extract PPI



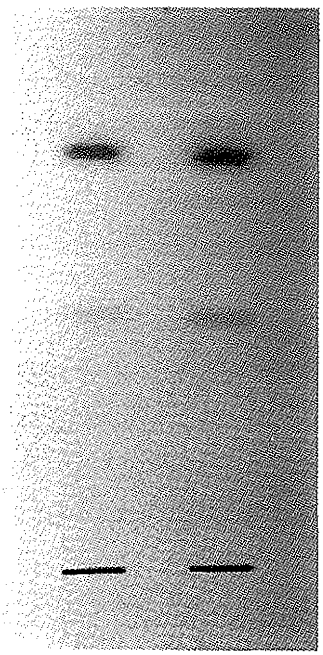
(Under UV light at 254 nm)

(IPRS1 = Silybin A IPRS, IPRS2 = Silybin B IPRS, IPRS3 = Isosilybin A IPRS and IPRS4 = Isosilybin B IPRS)

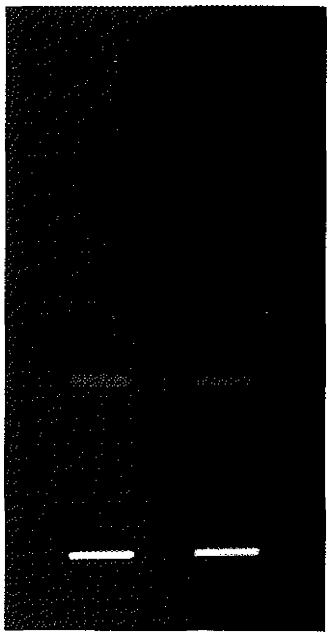
TLC Chromatogram of Silybum marianum Dry Extract

UNAUTHORISED COPY

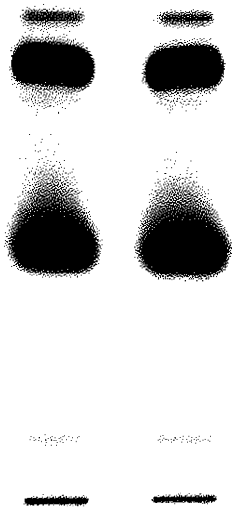
Ajwain



IPBRS TEST
(Under UV light at 254 nm)



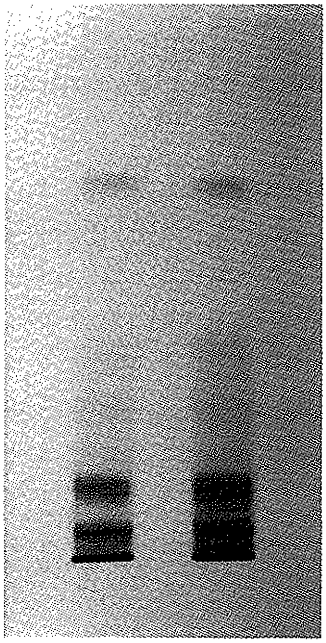
IPBRS TEST
(Under UV light at 366 nm)



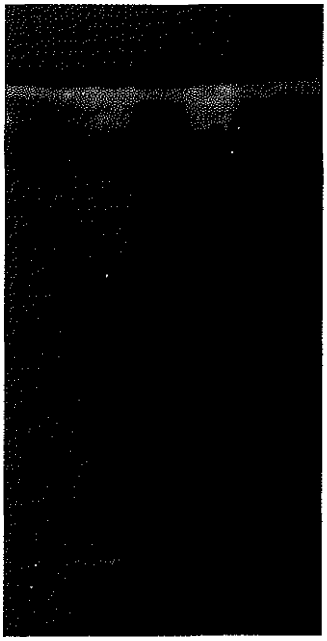
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Ajwain

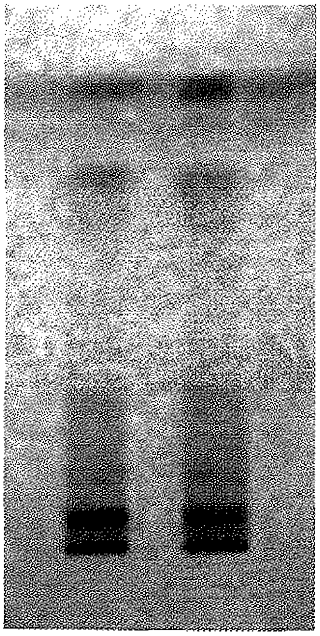
Amalaki



IPBRS TEST
(Under UV light at 254 nm)



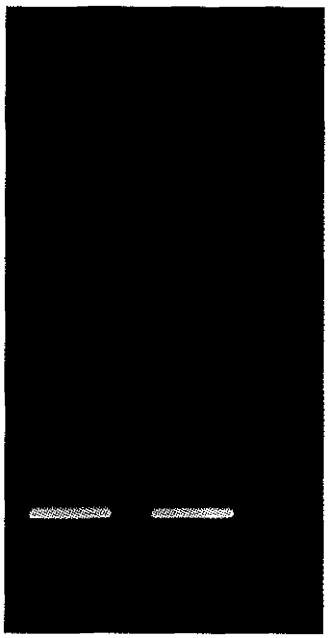
IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Amalaki

Amaltas

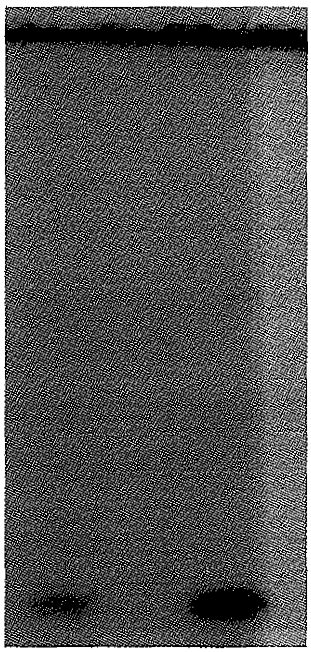


IPBRS TEST

(Under UV light at 366 nm)

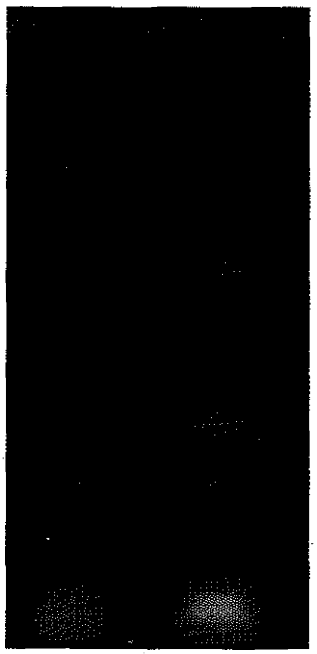
TLC Chromatogram of Amaltas

Amarbel



IPBRS TEST

(Under UV light at 254 nm)



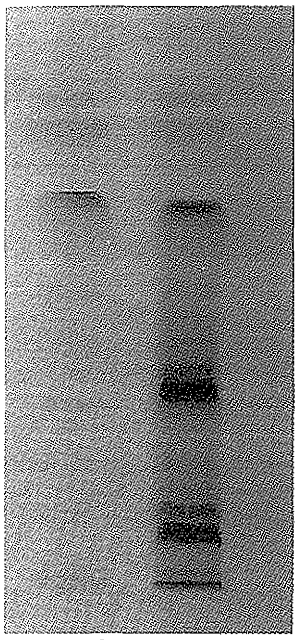
IPBRS TEST

(Under UV light at 366 nm)

TLC Chromatogram of Amarbel

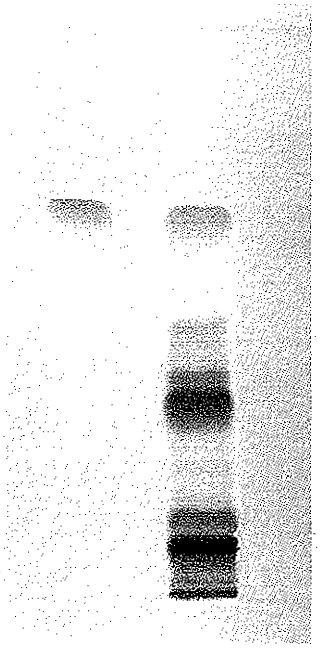
UNAUTHORISED COPY

Amla Juice Powder



IPBRS TEST

(Under UV light at 254 nm)



IPBRS TEST

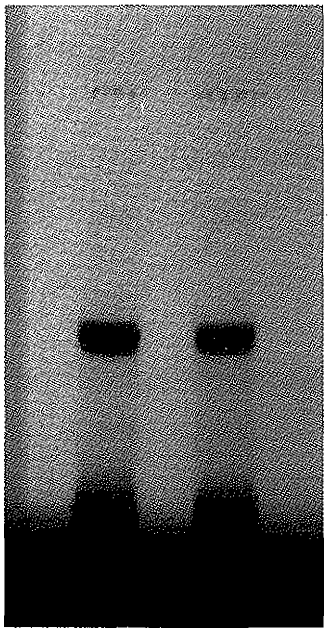
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)

IPBRS TEST

(Under day light after spraying with anisaldehyde sulphuric acid reagent)

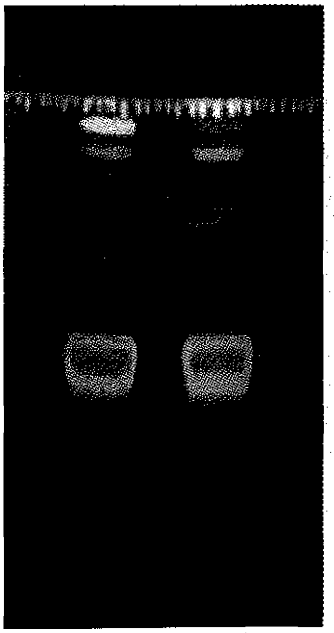
TLC Chromatogram of Amla Juice Powder

Amra



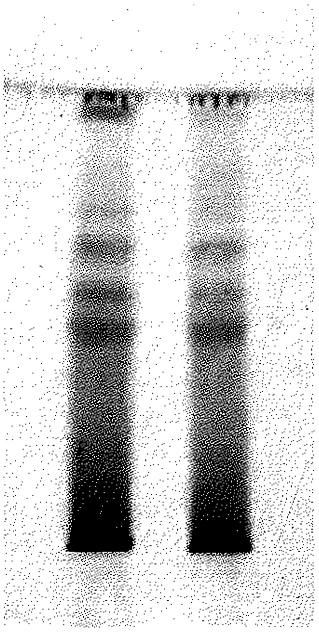
IPBRS TEST

(Under UV light at 254 nm)



IPBRS TEST

(Under UV light at 366 nm)



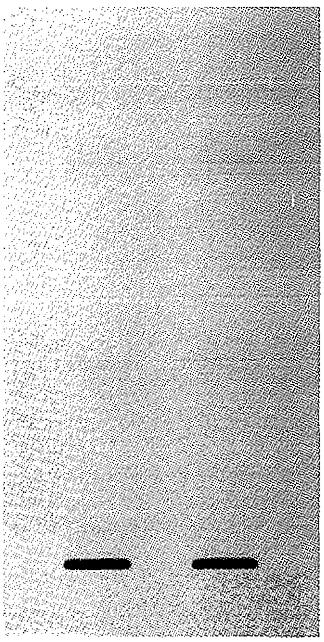
IPBRS TEST

(Under day light after spraying with vanillin glacial acetic acid reagent)

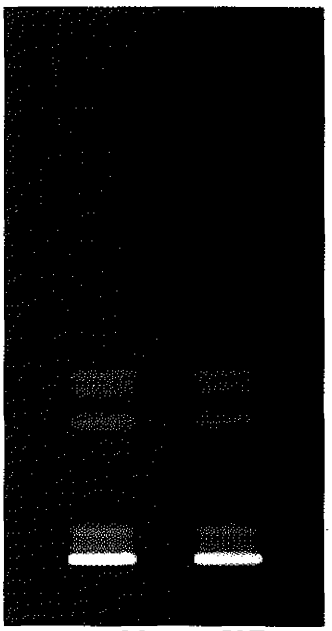
TLC Chromatogram of Amra

UNAUTHORISED COPY

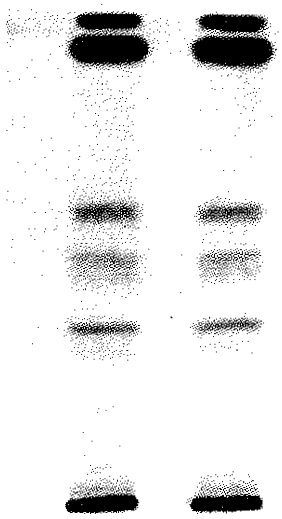
Anantmula



IPBRS TEST
(Under UV light at 254 nm)



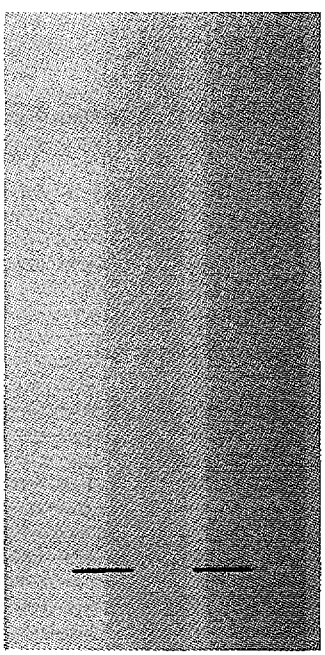
IPBRS TEST
(Under UV light at 366 nm)



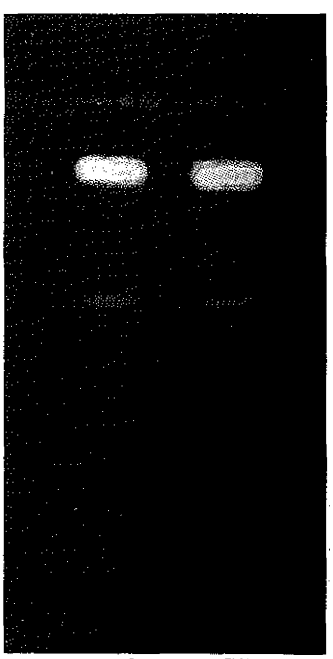
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Anantmula

Arjuna



IPBRS TEST
(Under UV light at 254 nm)

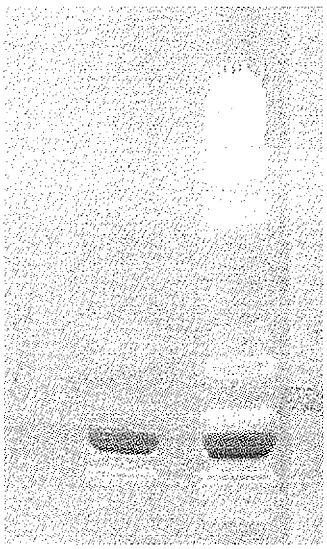


IPBRS TEST
(Under UV light at 366 nm)

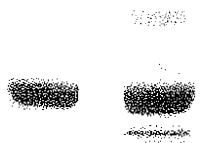
IPBRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

TLC Chromatogram of Arjuna

Arjuna Dry Extract



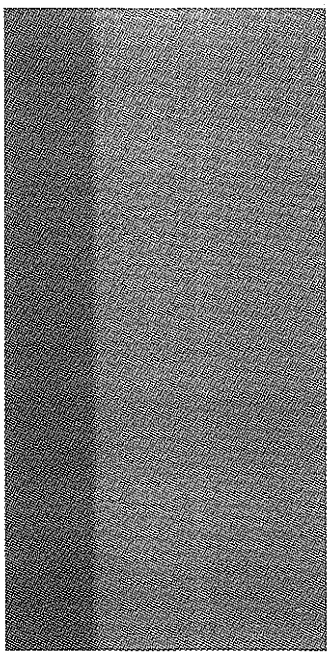
IPBRS TEST
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)



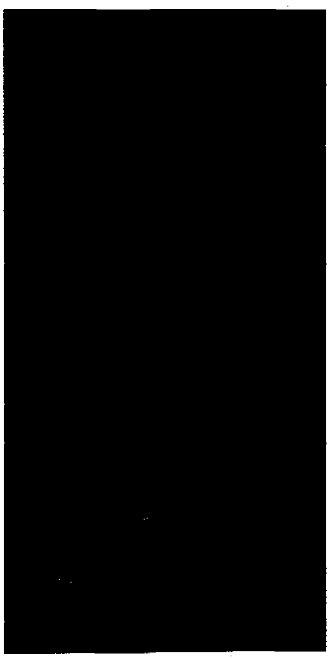
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Arjuna Dry Extract

Artemisia



IPBRS TEST
(Under UV light at 254 nm)



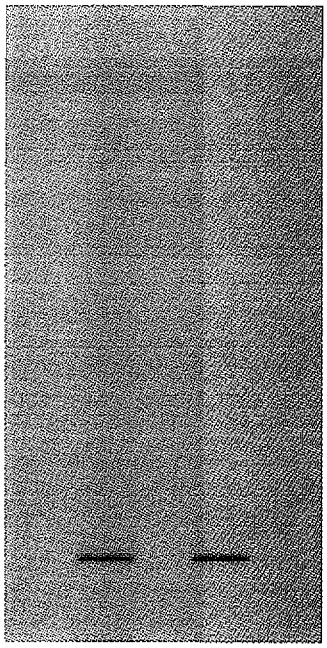
IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Artemisia

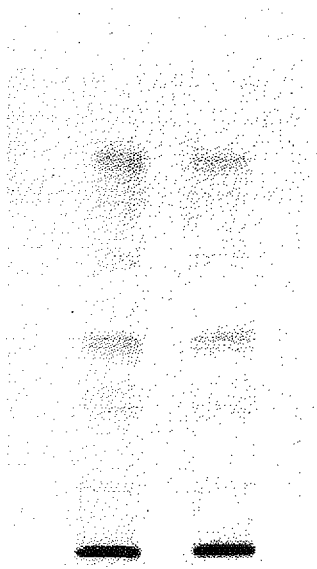
UNAUTHORISED COPY

Ashwagandha



IPBRS TEST
(Under UV light at 254 nm)

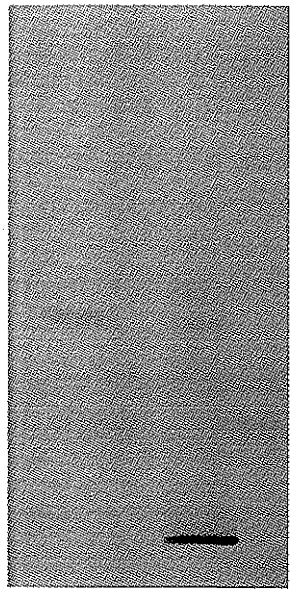
IPBRS TEST
(Under UV light at 366 nm)



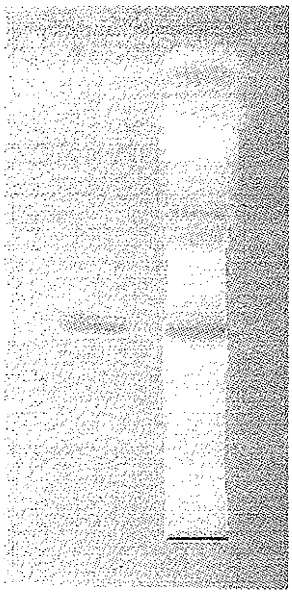
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Ashwagandha

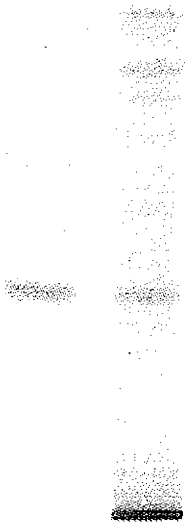
Ashwagandha Dry Extract



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)

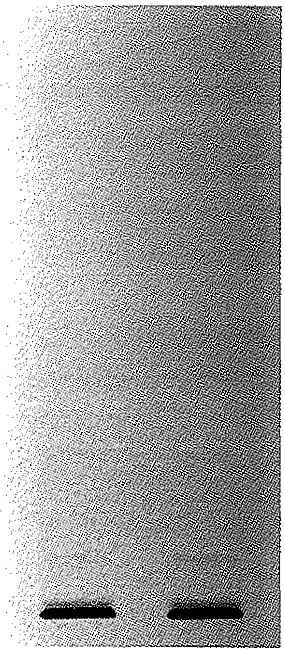


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

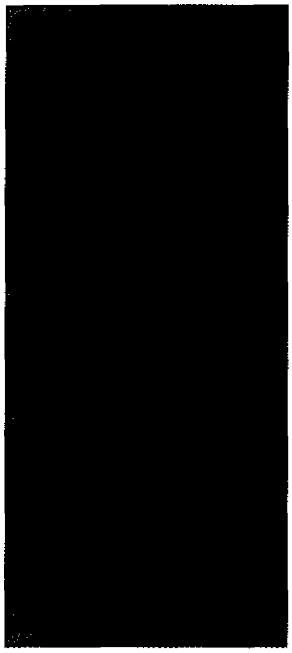
TLC Chromatogram of Ashwagandha Dry Extract

UNAUTHORISED COPY

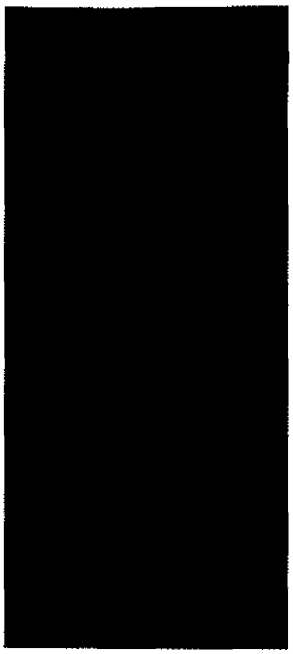
Asoka



IPBRS TEST
(Under UV light at 254 nm)



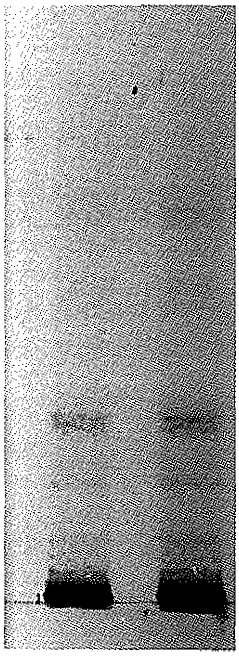
IPBRS TEST
(Under UV light at 366 nm)



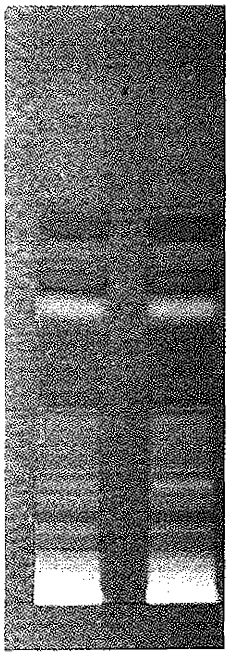
IPBRS TEST
(Under day light after spraying with methanolic sulphuric acid reagent)

TLC Chromatogram of Asoka

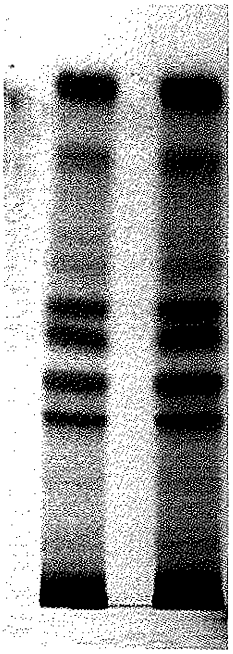
Asthisamhrta



IPBRS TEST
(Under UV light at 254 nm)



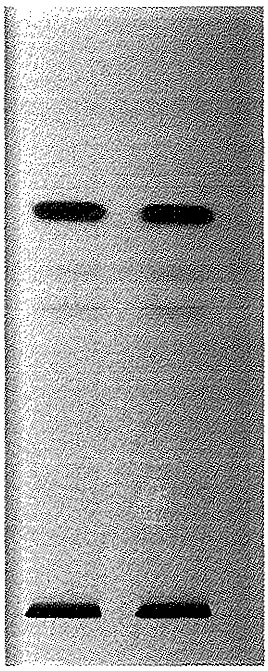
IPBRS TEST
(Under UV light at 366 nm)



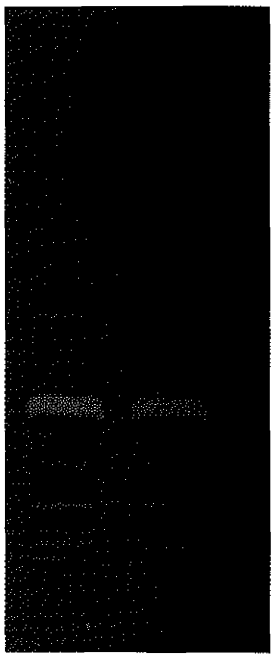
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Asthisamhrta

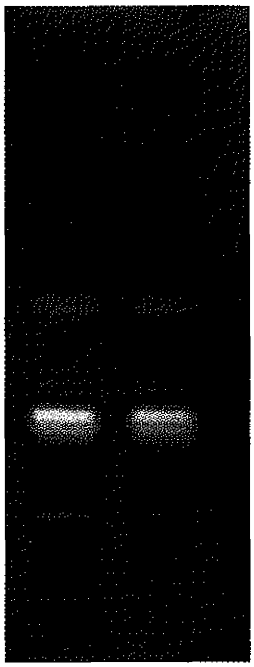
Bakuci



IPBRS TEST
(Under UV light at 254 nm)



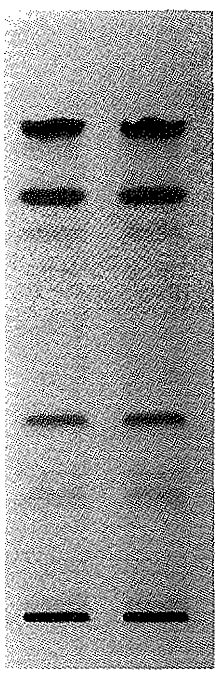
IPBRS TEST
(Under UV light at 366 nm)



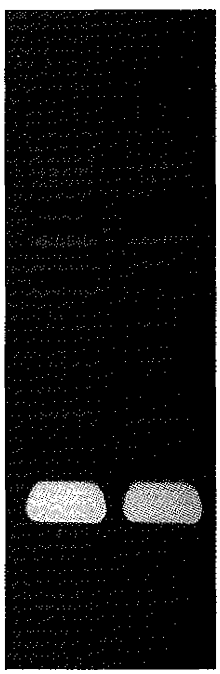
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Bakuci

Bala



IPBRS TEST
(Under UV light at 254 nm)

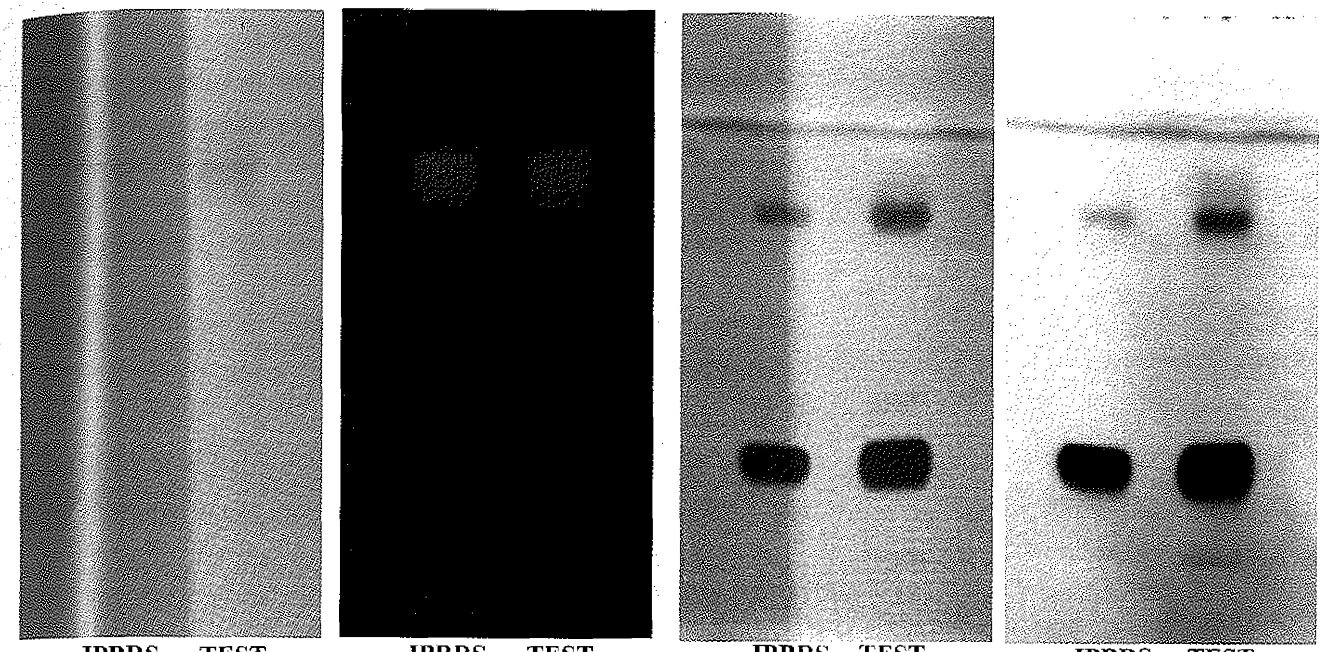


IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Bala

Belladonna Leaf



IPBRS TEST (Under UV light at 254 nm)

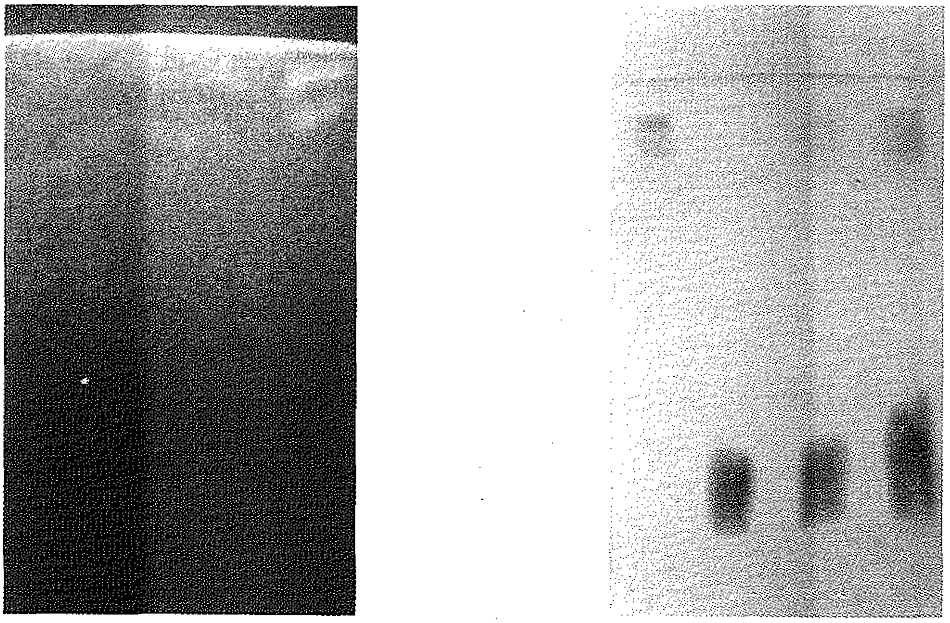
IPBRS TEST (Under UV light at 366 nm)

IPBRS TEST (Under day light after spraying with modified potassium iodobismuthate solution)

IPBRS TEST (Under day light after spraying with sodium nitrite solution)

TLC Chromatogram of Belladonna Leaf

Belladonna Dry Extract



IPRS2 IPRS1 IPRS1+2 TEST (Under UV light at 366 nm)

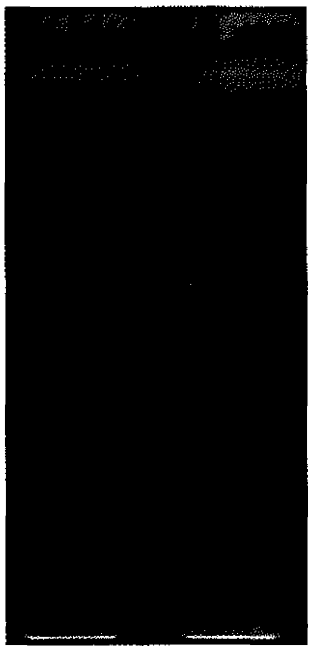
IPRS2 IPRS1 IPRS1+2 TEST (Under day light after spraying with modified potassium iodobismuthate solution)

(IPRS1 = Atropine sulphate IPRS and IPRS2 = Hyoscine hydrobromide IPRS)

TLC Chromatogram of Belladonna Dry Extract

UNAUTHORIS COPY

Belladonna Dry Extract Tablets

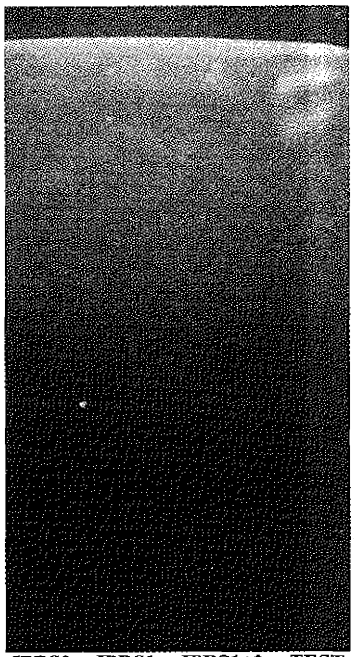


IPBRS TEST

(Under UV light at 366 nm)

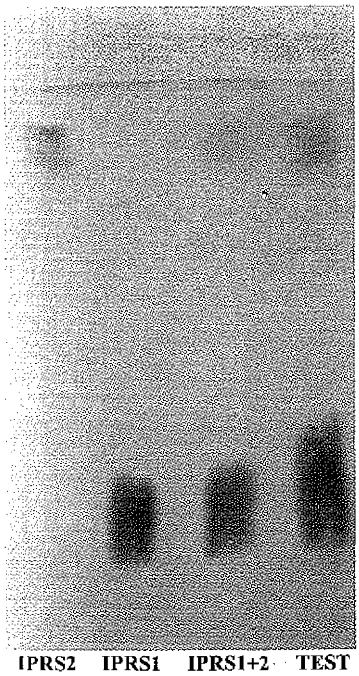
TLC Chromatogram of Belladonna Dry Extract Tablets

Belladonna Soft Extract



IPRS2 IPRS1 IPRS1+2 TEST

(Under UV light at 366 nm)



IPRS2 IPRS1 IPRS1+2 TEST

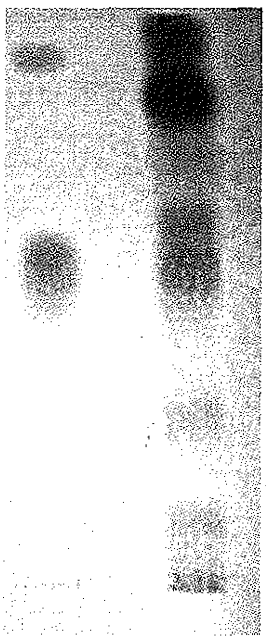
(Under day light after spraying with modified potassium iodobismuthate solution)

(IPRS1 = Atropine sulphate IPRS and IPRS2 = Hyoscine hydrobromide IPRS)

TLC Chromatogram of Belladonna Soft Extract

UNAUTHORISED COPY

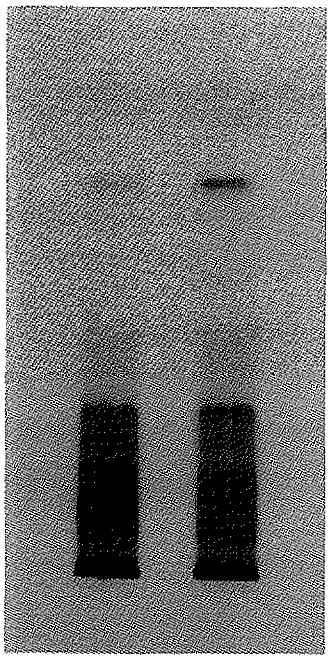
Belladonna Tincture



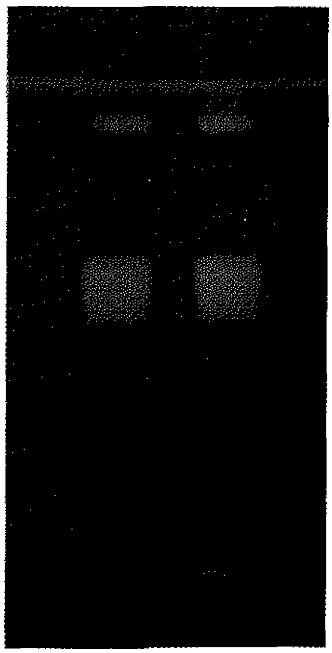
IPBRS TEST
(Under day light after spraying with potassium iodobismuthate solution)

TLC Chromatogram of Belladonna Tincture

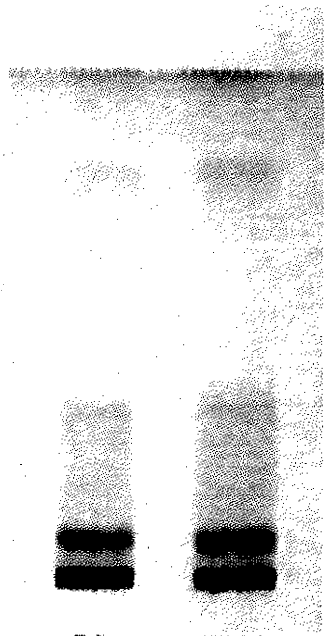
Bhibhitaki



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

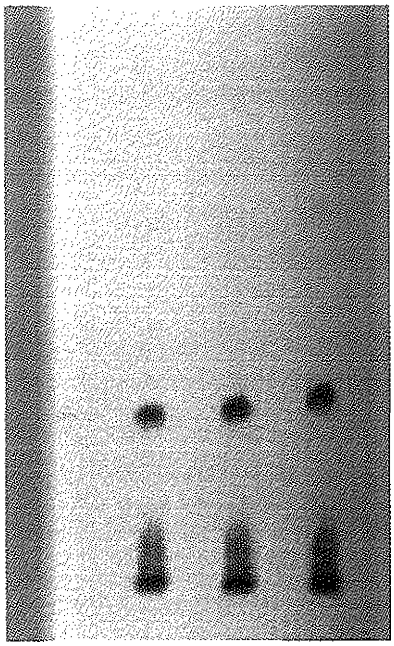


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Bhibhitaki

UNAUTHORISED COPY

Bhibhitaki Aqueous Extract

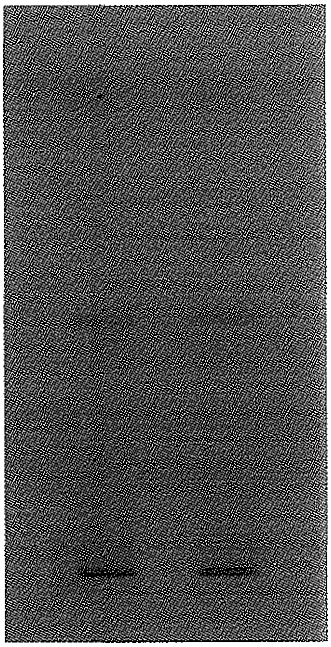


IPBRS TEST

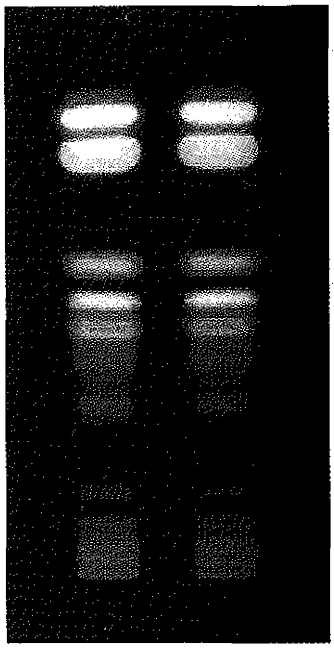
(Under UV light at 254 nm)

TLC Chromatogram of Bhibhitaki Aqueous Extract

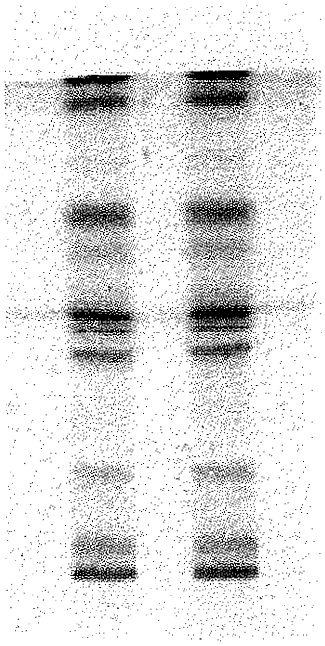
Bhringraj



IPBRS TEST
(Under UV light at 254 nm)



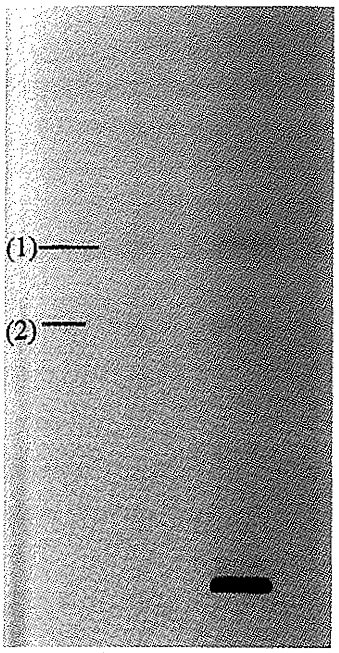
IPBRS TEST
(Under UV light at 366 nm)



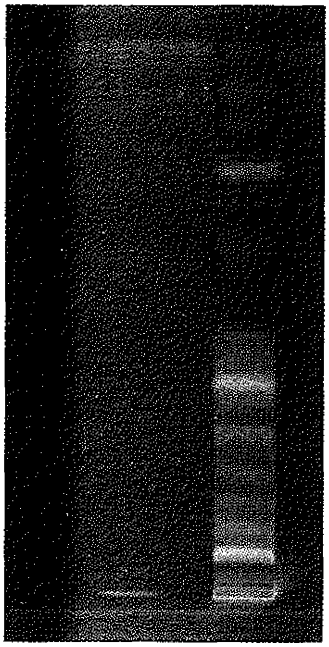
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Bhringraj

Bhuiamla Dry Extract



IPRS TEST
(Under UV light at 254 nm)



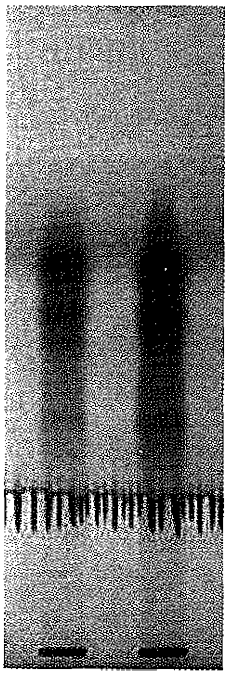
IPRS TEST
(Under UV light at 366 nm)

IPRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

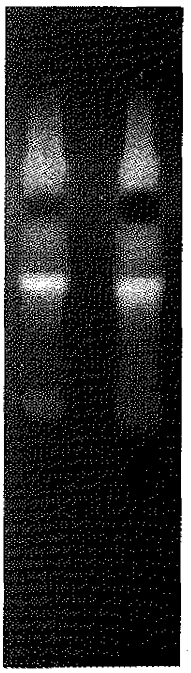
(IPRS1 = Phyllanthin IPRS and IPRS2 = Hypophyllanthin IPRS)

TLC Chromatogram of Bhuiamla Dry Extract

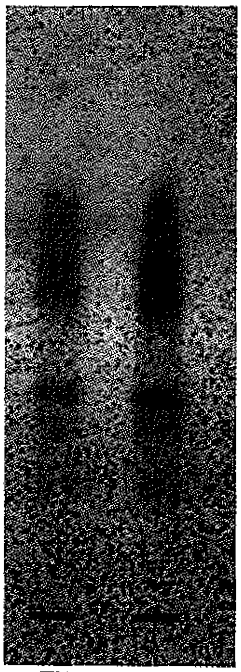
Birmi



IPBRS TEST
(Under UV light at 254 nm)



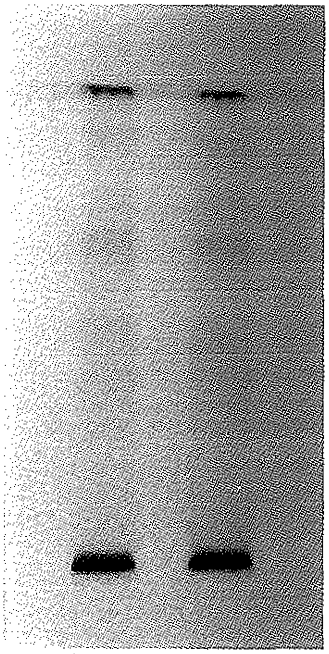
IPBRS TEST
(Under UV light at 366 nm)



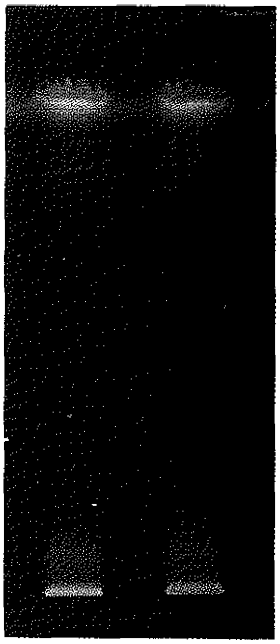
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Birmi

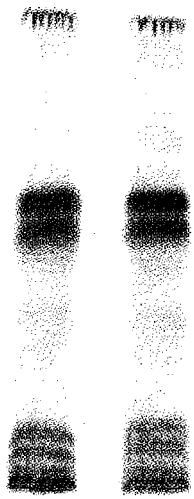
Brahmi



IPBRS TEST
(Under UV light at 254 nm)



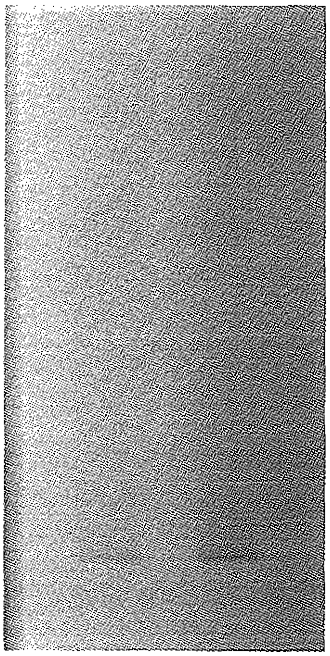
IPBRS TEST
(Under UV light at 366 nm)



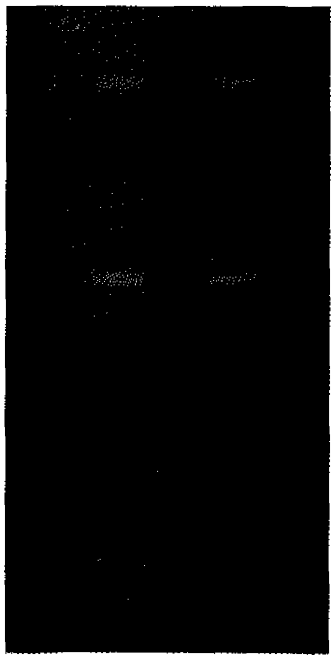
IPBRS TEST
(Under day light after spraying with 20 per cent, v/v methanolic sulphuric acid reagent)

TLC Chromatogram of Brahmi

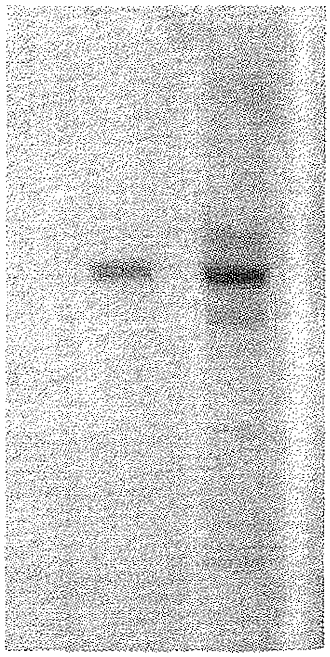
Brahmi Extract



IPBRS TEST
(Under UV light at 254 nm)



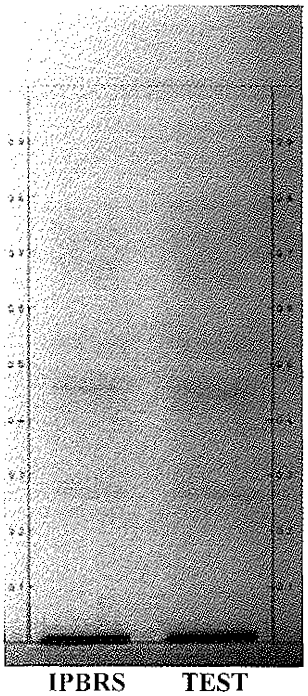
IPBRS TEST
(Under UV light at 366 nm after spraying with vanillin sulphuric acid solution)



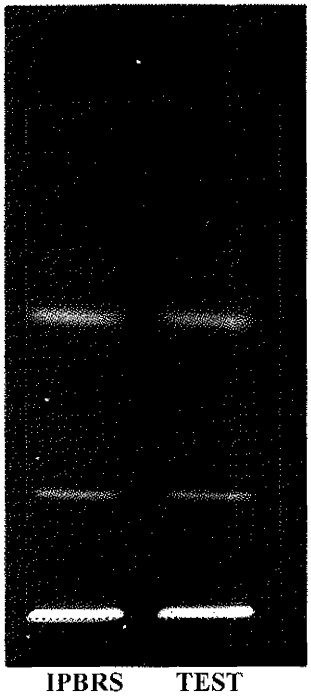
IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid solution)

TLC Chromatogram of Brahmi Extract

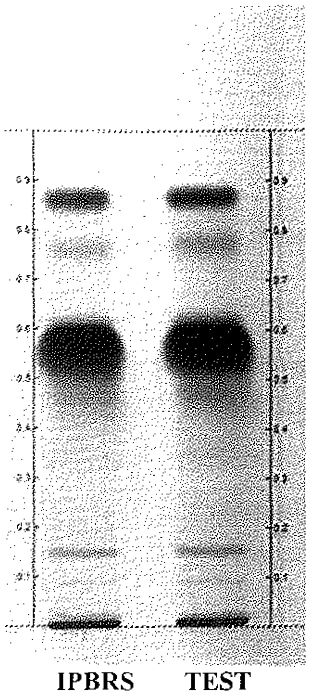
Chaulai



IPBRS TEST
(Under UV light at 254 nm)



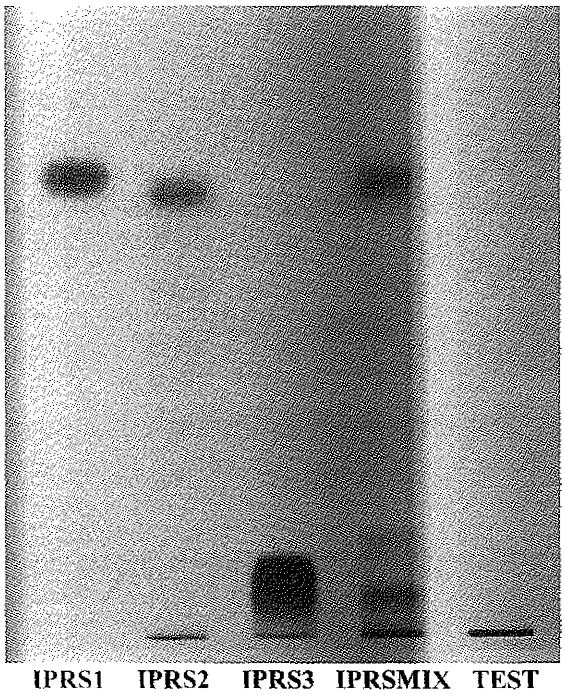
IPBRS TEST
(Under UV light at 366 nm)



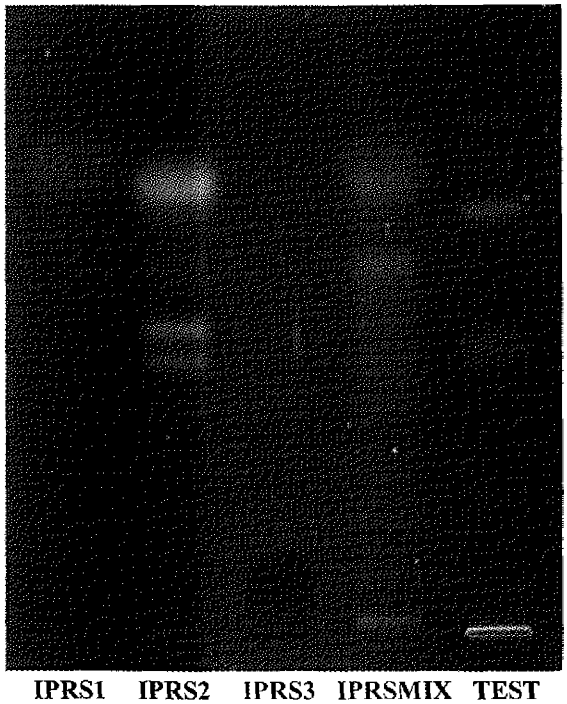
IPBRS TEST
(Under day light after spraying with iodine vapours)

TLC Chromatogram of Chaulai

Chitrak



IPRS1 IPRS2 IPRS3 IPRSMIX TEST
(Under UV light at 254 nm)



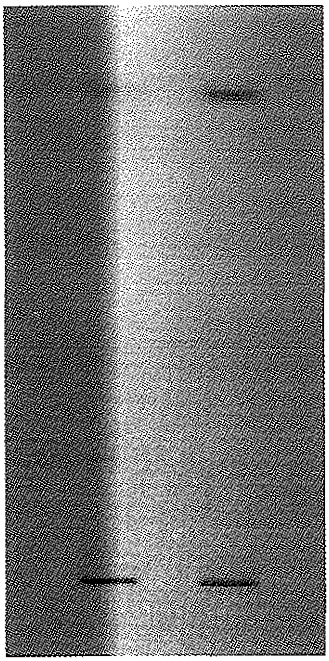
IPRS1 IPRS2 IPRS3 IPRSMIX TEST
(Under UV light at 366 nm)

(IPRS1 = Plumbagin IPRS, IPRS2 = Juglone IPRS and IPRS3 = Lawsone IPRS)

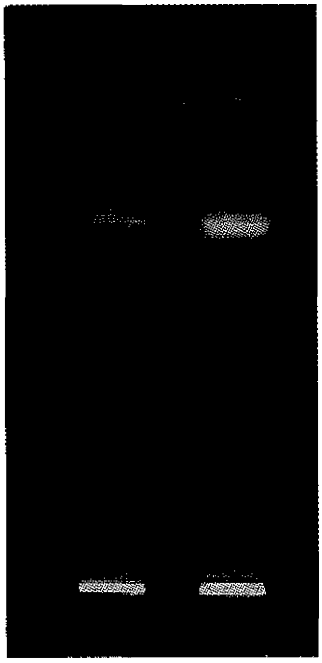
TLC Chromatogram of Chitrak

UNAUTHORISED

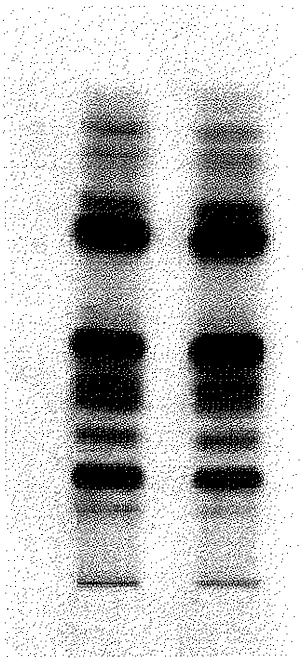
Coleus



IPBRS TEST
(Under UV light at 254 nm)



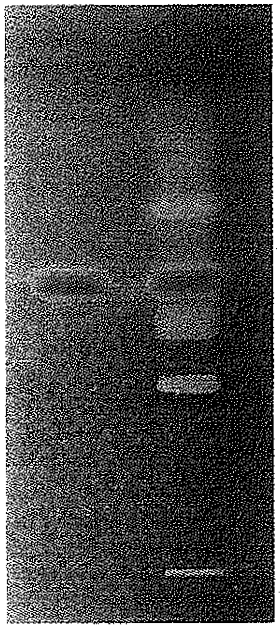
IPBRS TEST
(Under UV light at 366 nm)



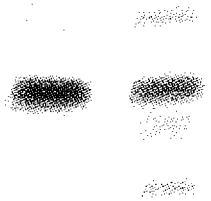
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Coleus

Coleus Dry Extract



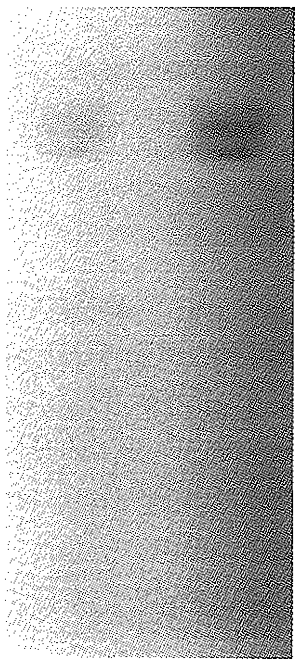
IPRS TEST
(Under UV light at 366 after spraying with anisaldehyde sulphuric acid reagent)



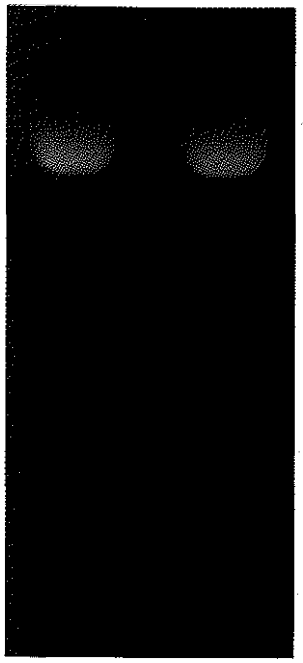
IPRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Coleus Dry Extract

Daruharidra Roots



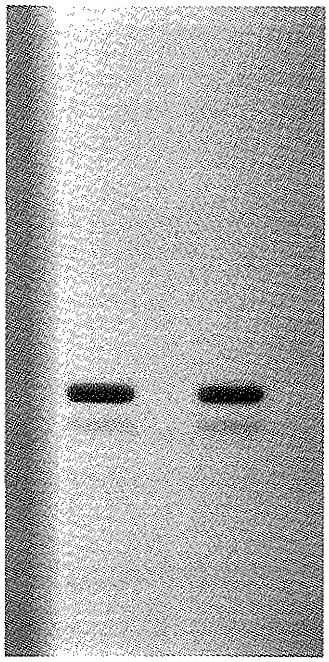
IPRS TEST
(Under UV light at 254 nm)



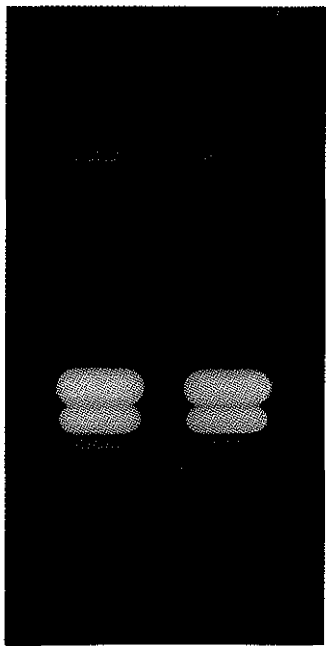
IPRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Daruharidra Roots

Daruharidra Roots and Stems



IPBRS TEST
(Under UV light at 254 nm)



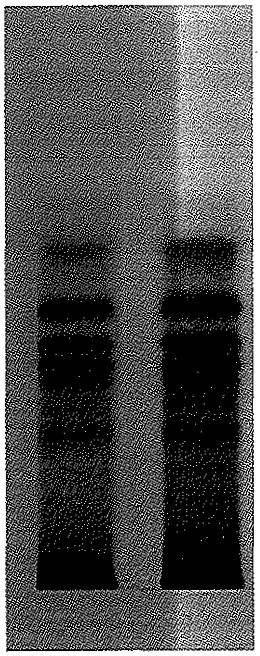
IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light)

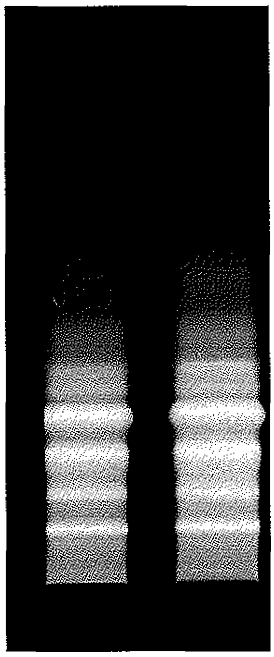
TLC Chromatogram of Daruharidra Roots and Stems

UNAUTHORISED COPY

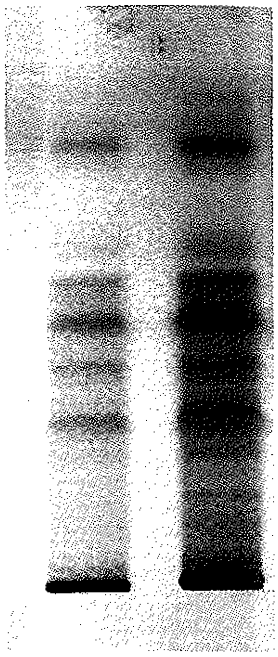
Draksha



IPBRS TEST
(Under UV light at 254 nm)



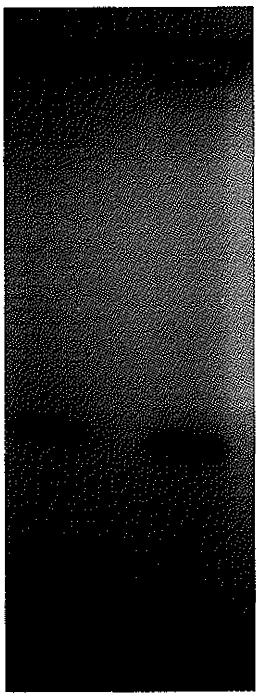
IPBRS TEST
(Under UV light at 366 nm)



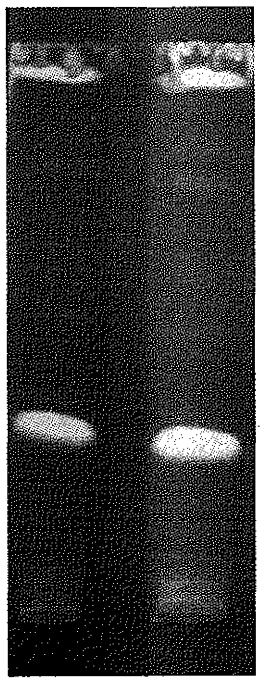
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Draksha

Ergot



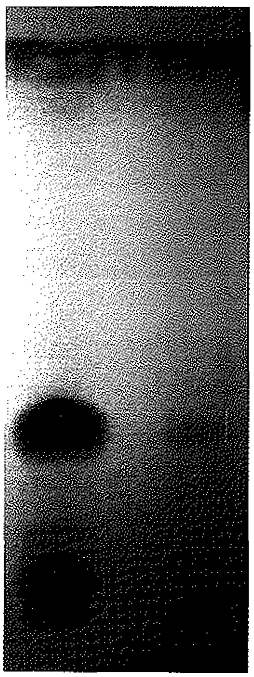
IPBRS TEST
(Under UV light at 254 nm)



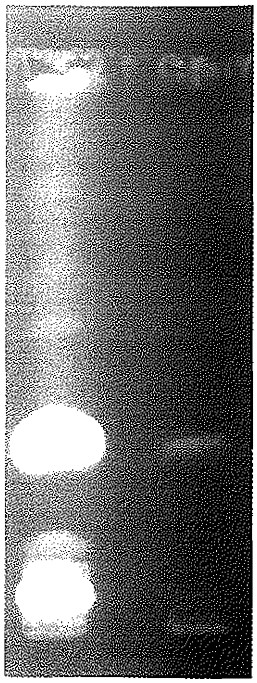
IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Ergot

Prepared Ergot



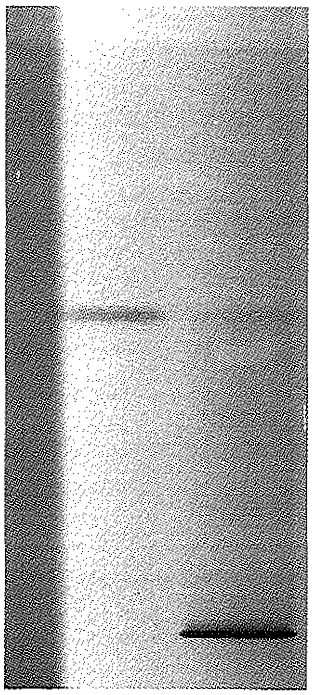
IPRS TEST
(Under UV light at 254 nm)



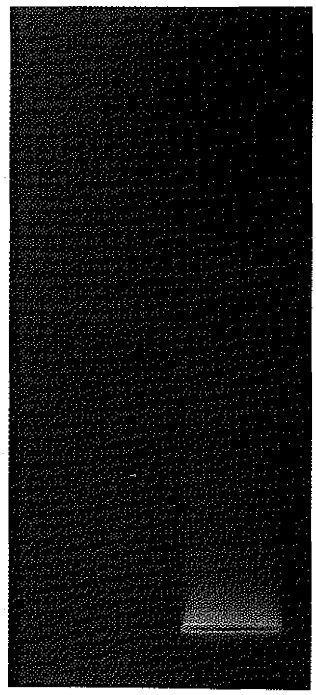
IPRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Prepared Ergot

Garcinia



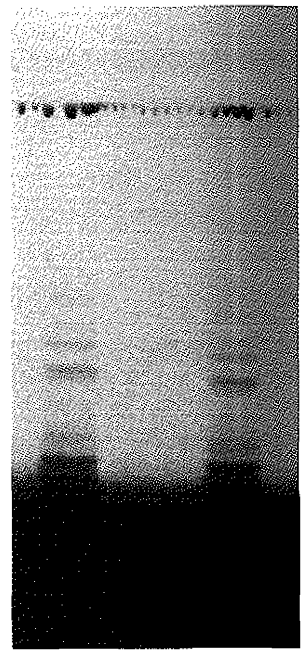
IPRS TEST
(Under UV light at 254 nm)



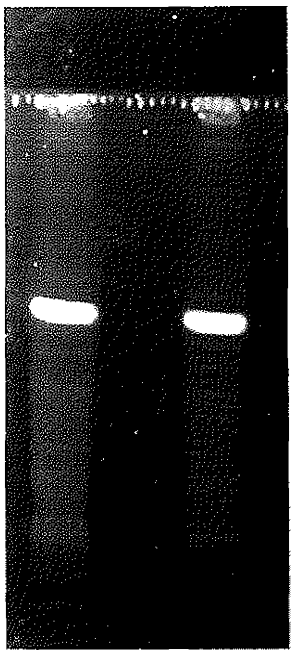
IPRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Garcinia

Ginkgo Dry Extract



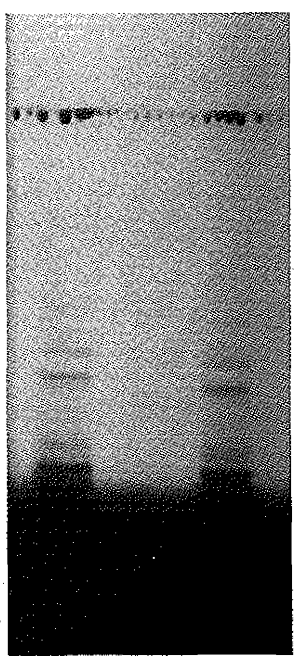
IPBRS TEST
(Under UV light at 254 nm)



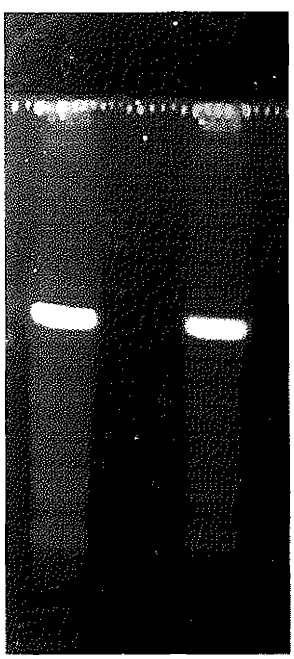
IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Ginkgo Dry Extract

Ginkgo Tablets



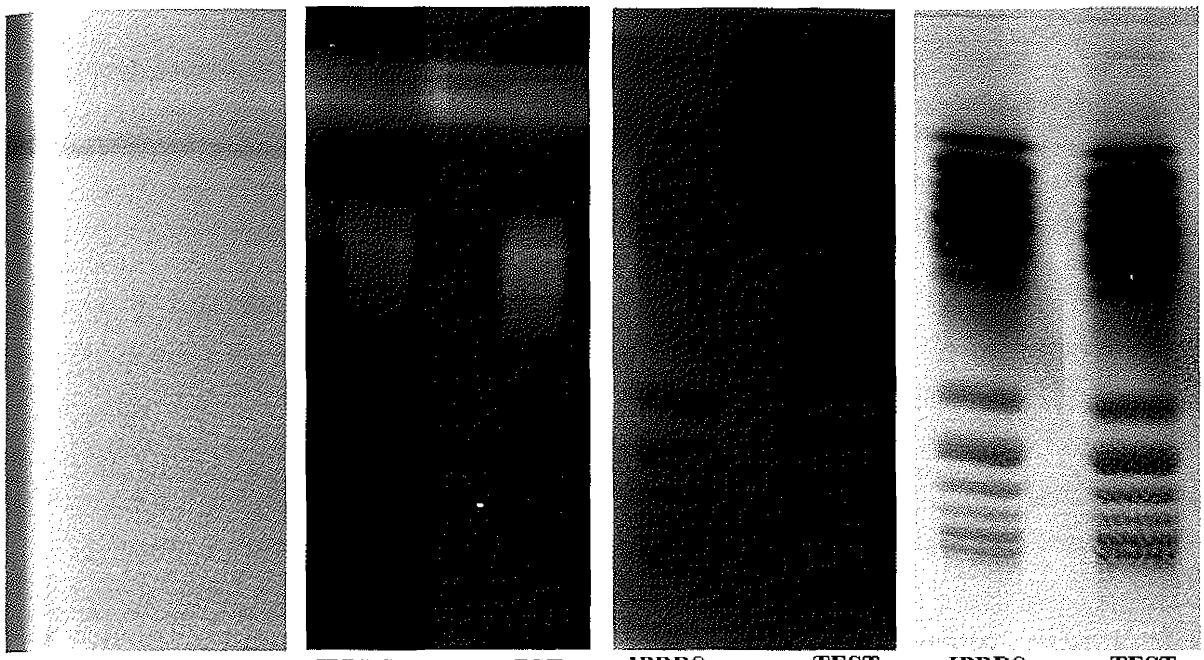
IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Ginkgo Tablets

Ginseng Dry Extract



IPBRS TEST (Under UV light at 254 nm)

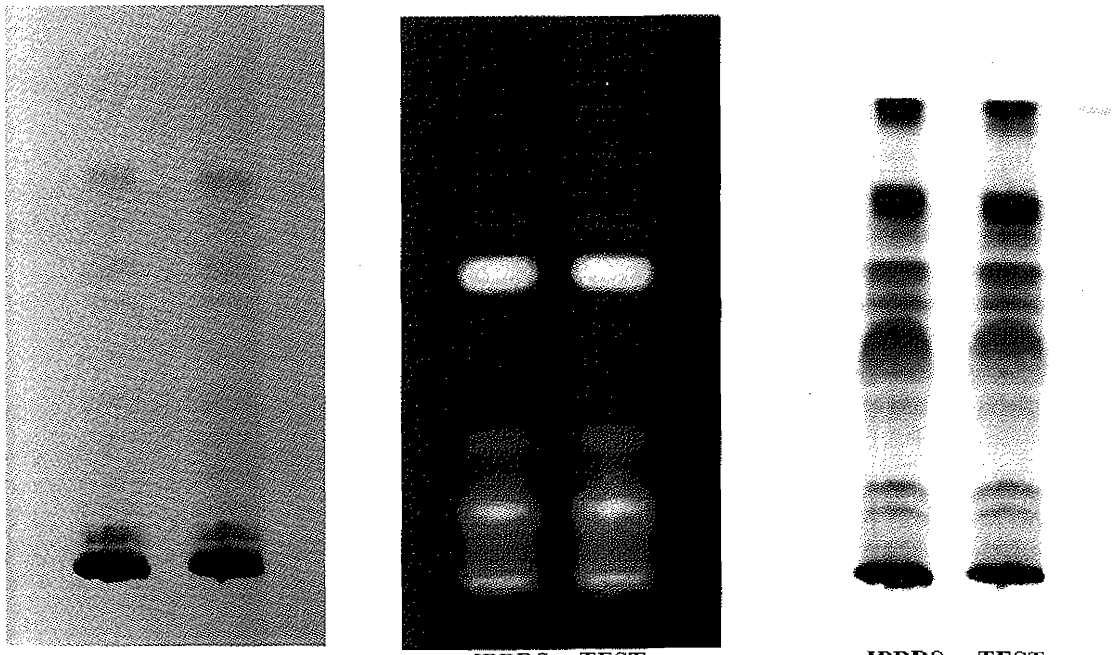
IPBRS TEST (Under UV light at 366 nm)

IPBRS TEST (Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)

IPBRS TEST (Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Ginseng Dry Extract

Gokhru



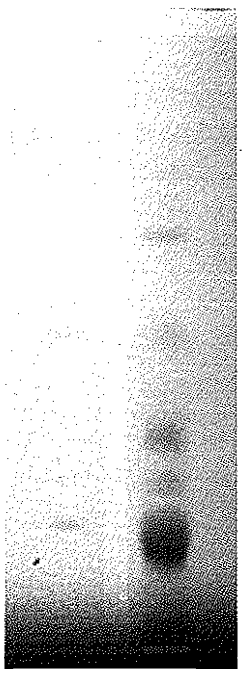
IPBRS TEST (Under UV light at 254 nm)

IPBRS TEST (Under UV light at 366 nm)

IPBRS TEST (Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Gokhru

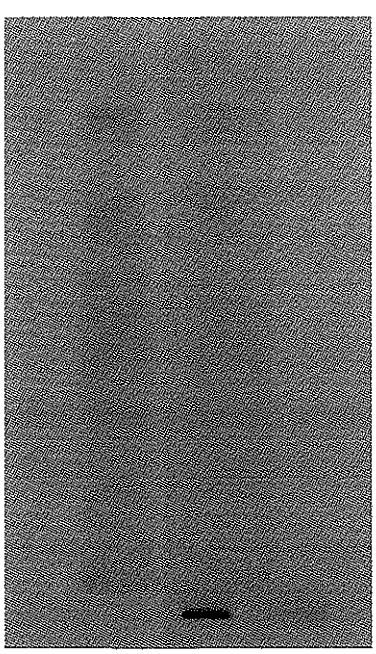
Green Coffee Bean Extract



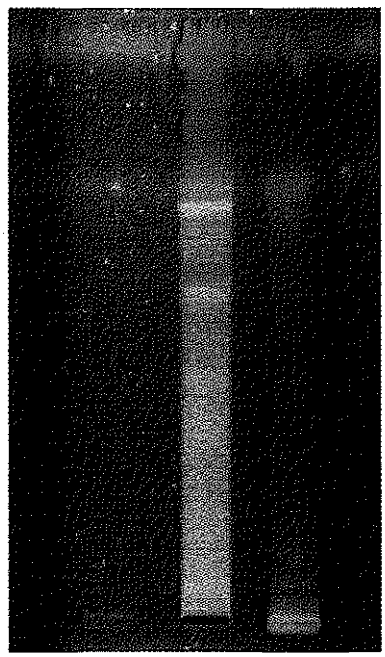
IPRS TEST
(Under UV light at 254 nm)

TLC Chromatogram of Green Coffee Bean Extract

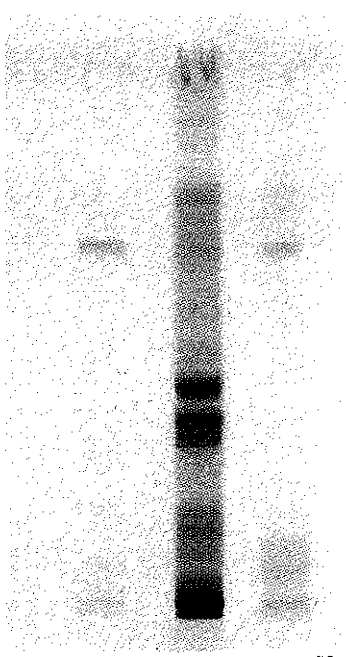
Gudmar Dry Extract



IPRS TEST1 TEST2
(Under UV light at 254 nm)



IPRS TEST1 TEST2
(Under UV light at 366 nm)

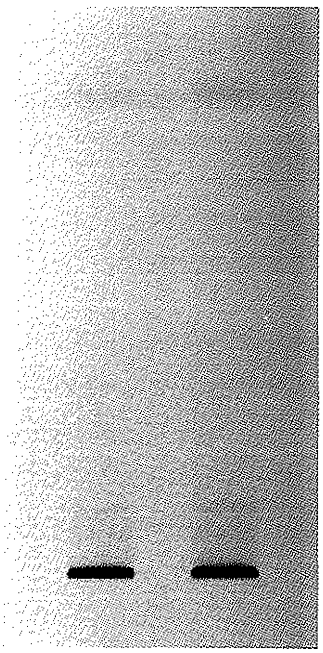


IPRS TEST1 TEST2
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

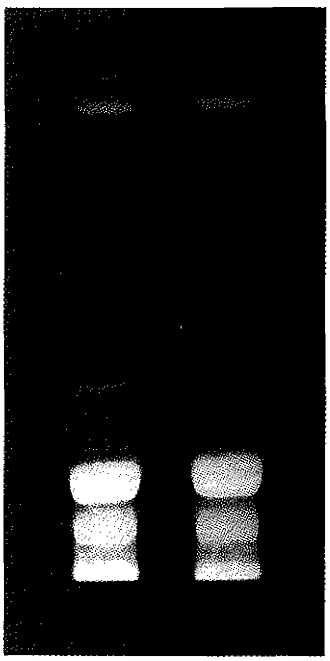
TLC Chromatogram of Gudmar Dry Extract

AUTHORISED COPY

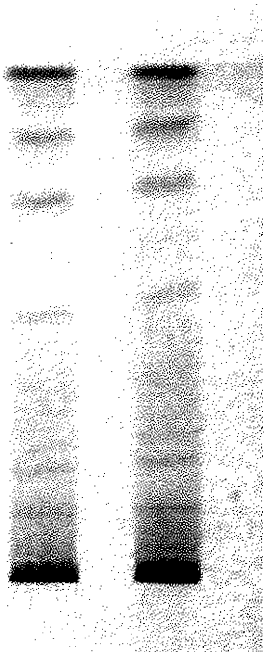
Guduchi



IPBRS TEST
(Under UV light at 254 nm)



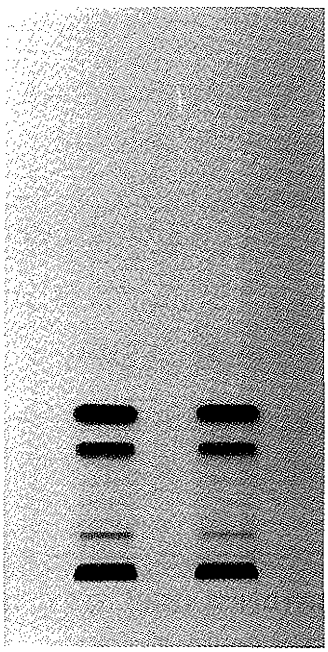
IPBRS TEST
(Under UV light at 366 nm)



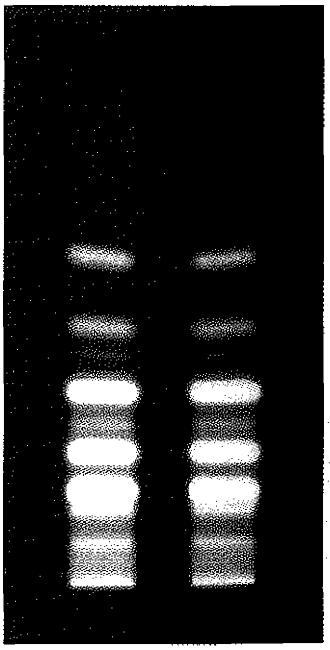
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Guduchi

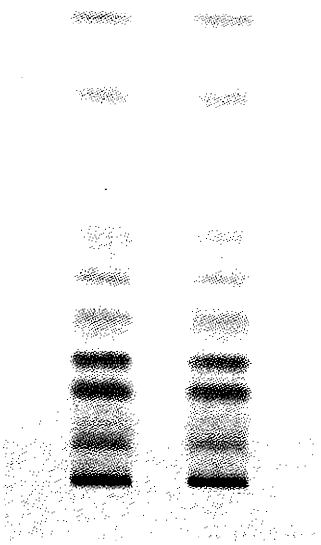
Guggul Resin



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

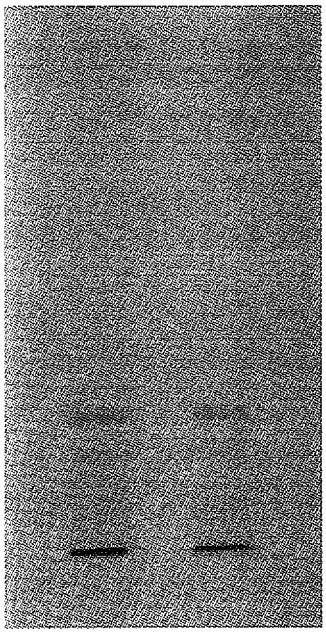


IPBRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

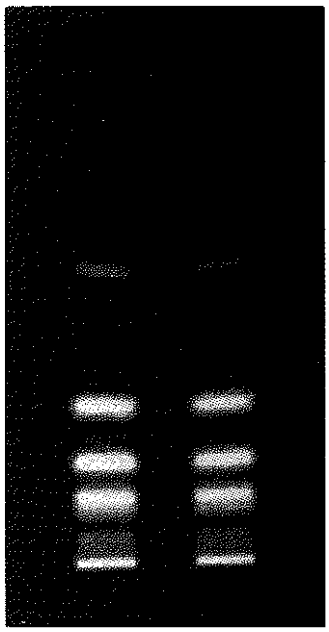
TLC Chromatogram of Guggul Resin

MONOGRAPH COPY

Guggulipid



IPBRS TEST
(Under UV light at 254 nm)

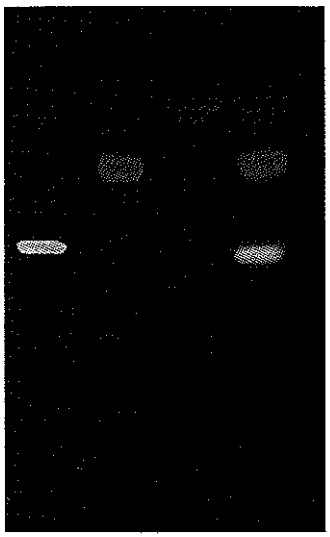


IPBRS TEST
(Under UV light at 366 nm)

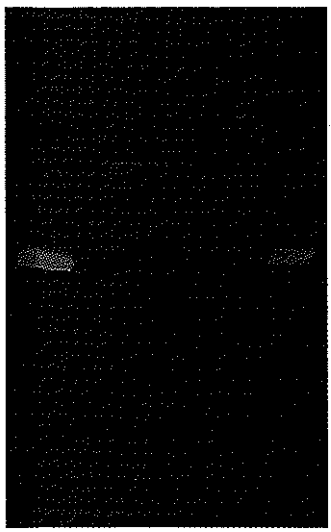
IPBRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

TLC Chromatogram of Guggulipid

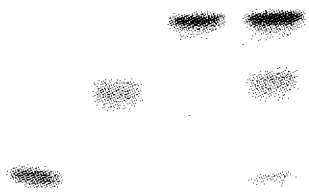
Haridra Dry Extract



IPRS1 IPRS2 IPRS3 TEST
(Under UV light at 254 nm)



IPRS1 IPRS2 IPRS3 TEST
(Under UV light at 366 nm)



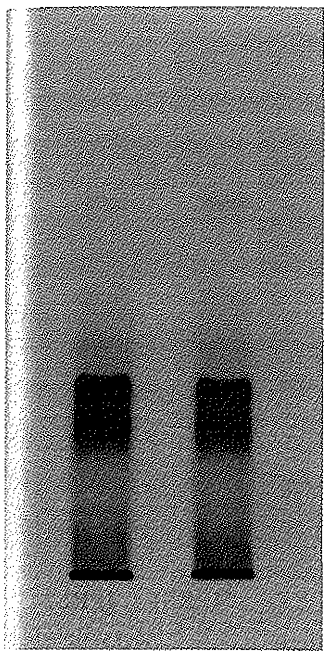
IPRS1 IPRS2 IPRS3 TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

IPRS1 = Bisdemethoxycurcumin IPRS, IPRS2 = Demethoxycurcumin IPRS and IPRS3 = Curcumin IPRS

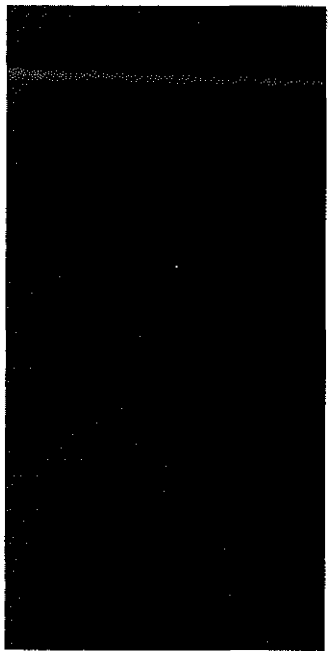
TLC Chromatogram of Haridra Dry Extract

UNAUTHORISED COPY

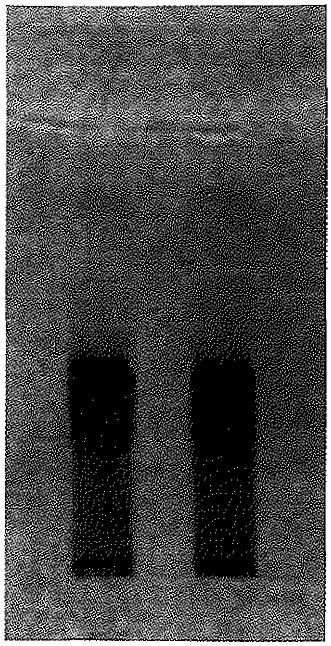
Haritaki



IPBRS TEST
(Under UV light at 254 nm)



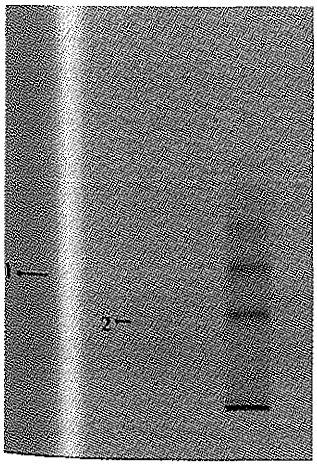
IPBRS TEST
(Under UV light at 366 nm)



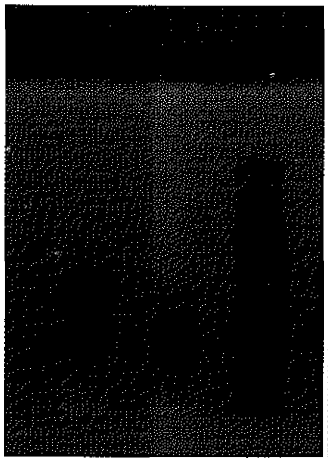
IPBRS TEST
(Under day light after spraying with 10 per cent, v/v ferric chloride solution)

TLC Chromatogram of Haritaki

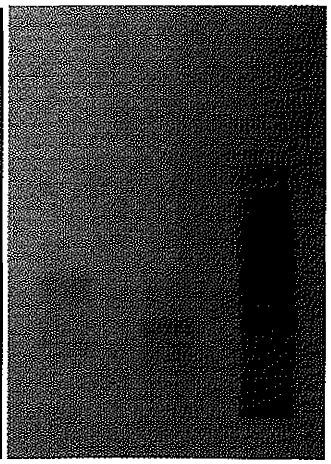
Haritaki Extract



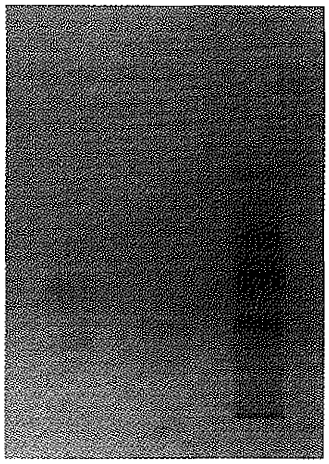
IPRS1 IPRS2 TEST
(Under UV light at 254 nm)



IPRS1 IPRS2 TEST
(Under UV light at 366 nm)



IPRS1 IPRS2 TEST
(Under visible light before spraying)



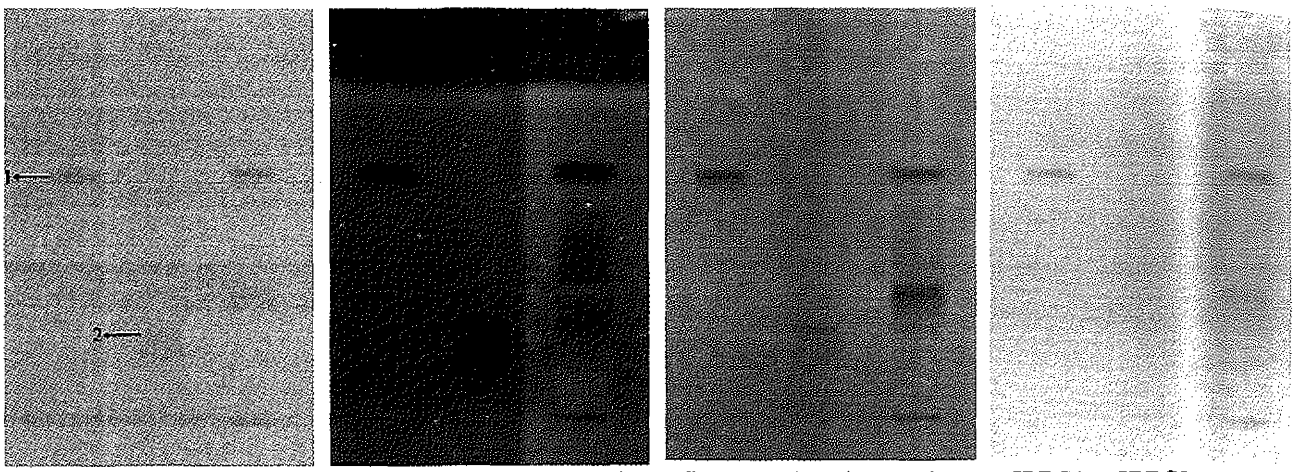
IPRS1 IPRS2 TEST
(Under visible light after spraying with ferric chloride reagent)

(IPRS1 = Chebulinic acid IPRS and IPRS2 = Chebulagic acid IPRS)

TLC Chromatogram of Haritaki Extract

UNAUTHORISED COPY

Haritaki Aqueous Extract

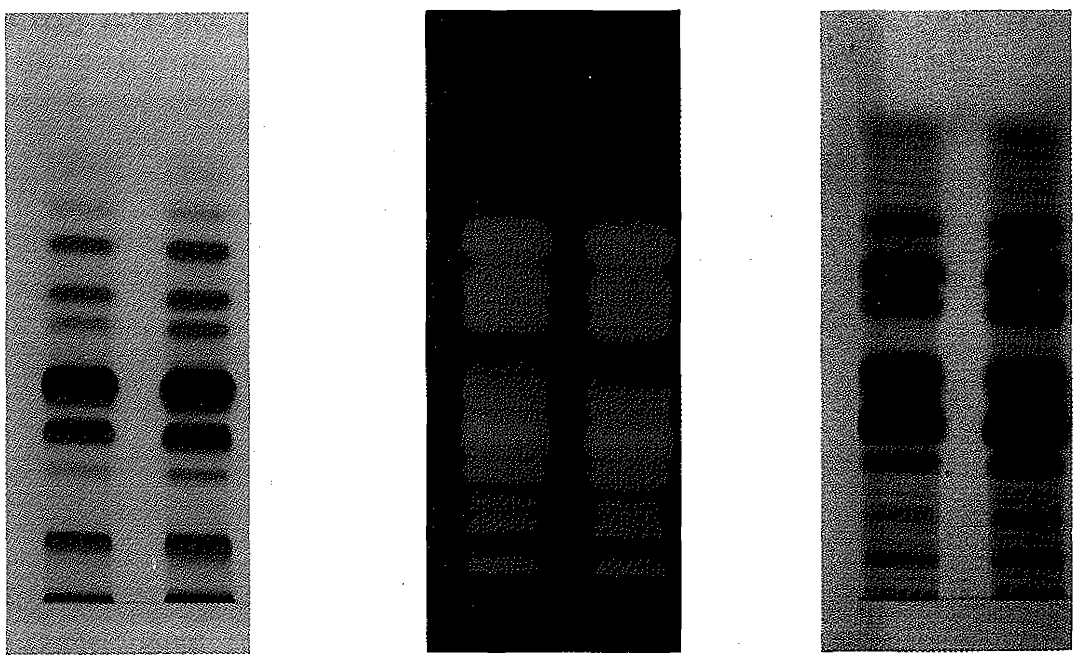


IPRS1 IPRS2 TEST (Under UV light at 254 nm) | IPRS1 IPRS2 TEST (Under UV light at 366 nm) | IPRS1 IPRS2 TEST (Under visible light before spraying) | IPRS1 IPRS2 TEST (Under visible light after spraying with ferric chloride reagent)

(IPRS1 = Gallic acid IPRS and IPRS2 = Chebulagic acid IPRS)

TLC Chromatogram of Haritaki Aqueous Extract

Hingu

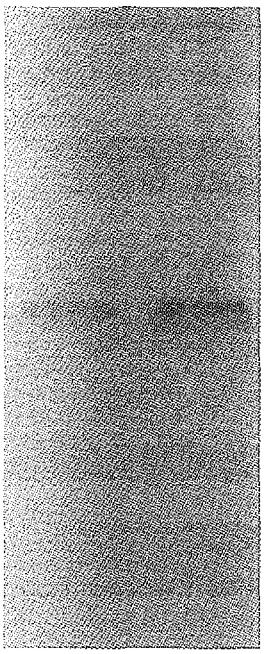


IPBRS TEST (Under UV light at 254 nm) | IPBRS TEST (Under UV light at 366 nm) | IPBRS TEST (Under day light after spraying with anisaldehyde sulphuric acid reagent)

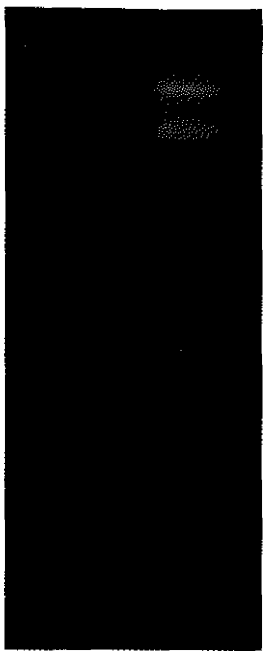
TLC Chromatogram of Hingu

UNAUTHORISED COPY

Horse Chestnut Dry Extract



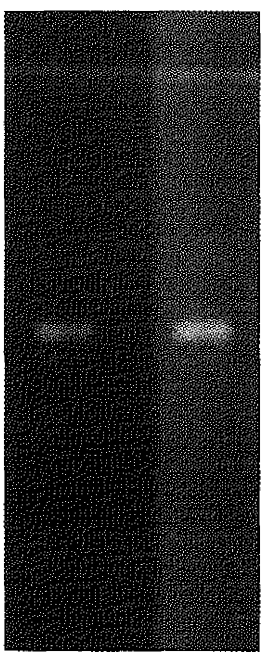
IPBRS TEST
(Under UV light at 254 nm)



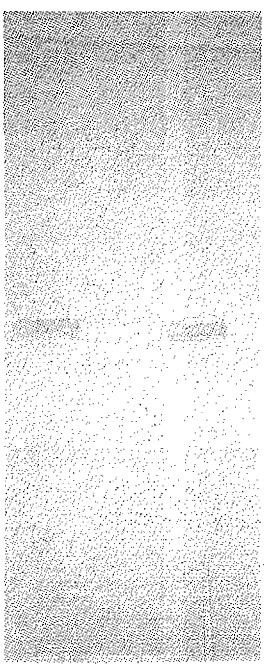
IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Horse Chestnut Dry Extract

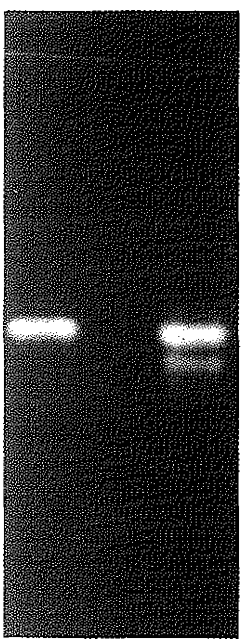
Ipecac Tincture



IPRS TEST
(Under UV light at 366 nm)



IPRS TEST
(Under daylight after spraying with iodine in ethanol 95 per cent v/v solution)

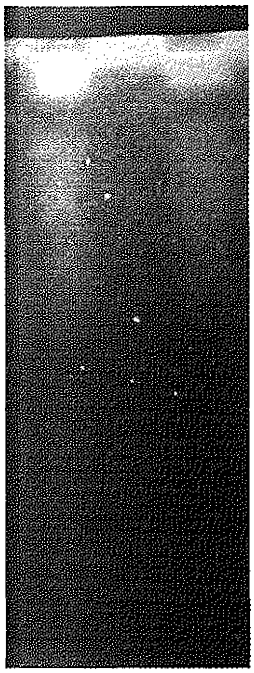


IPRS TEST
(Under UV light at 366 nm after spraying with iodine in ethanol 95 per cent v/v solution)

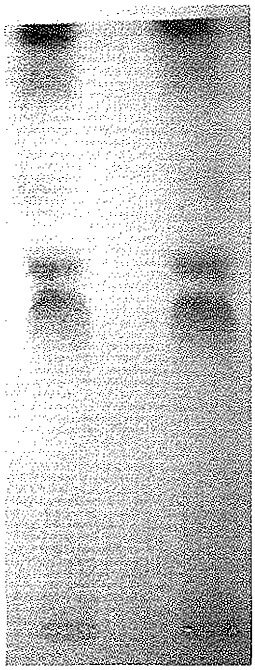
TLC Chromatogram of Ipecac Tincture

UNCONTROLLED

Ivy Leaf



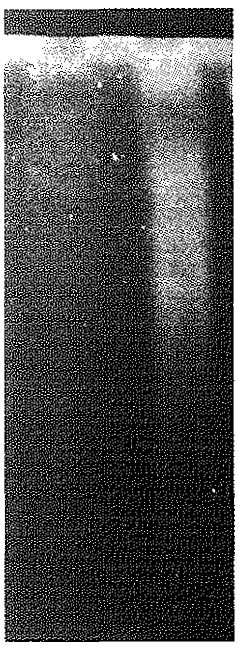
IPBRS TEST
(Under UV light at 366 nm)



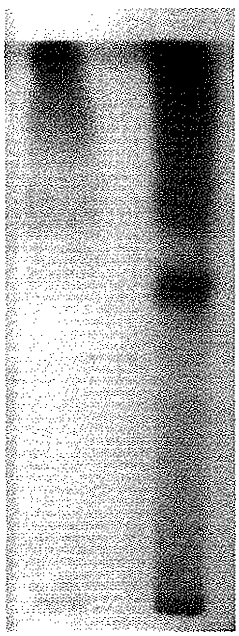
IPBRS TEST
(Under daylight after spraying with 10 per cent alcoholic sulphuric acid)

TLC Chromatogram of Ivy Leaf

Ivy Leaf Dry Extract



IPBRS TEST
(Under UV light at 366 nm)

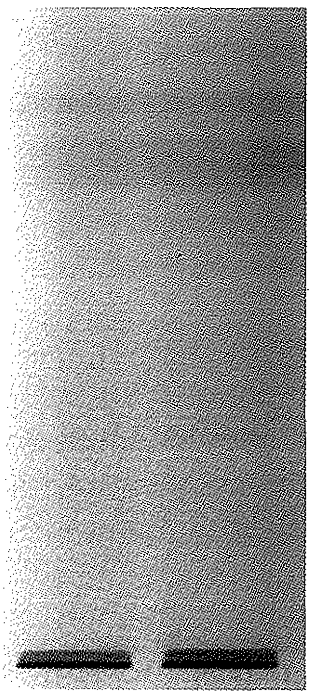


IPBRS TEST
(Under daylight after spraying with 10 per cent alcoholic sulphuric acid)

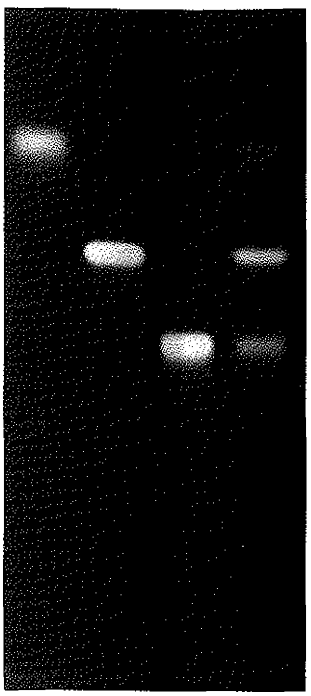
TLC Chromatogram of Ivy Leaf Dry Extract

UNAUTHORISED Copy

Jangali Haldi



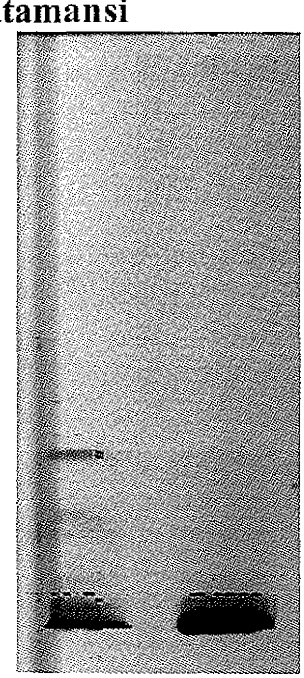
IPBRS TEST
(Under UV light at 254 nm)



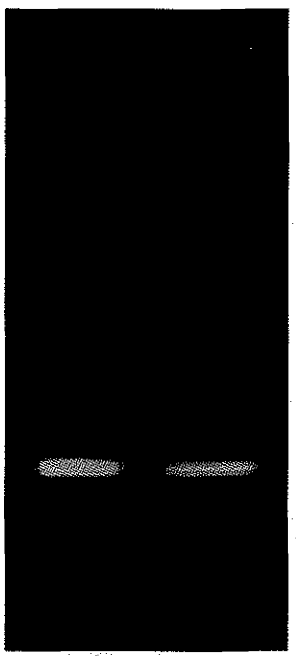
IPRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Jangali Haldi

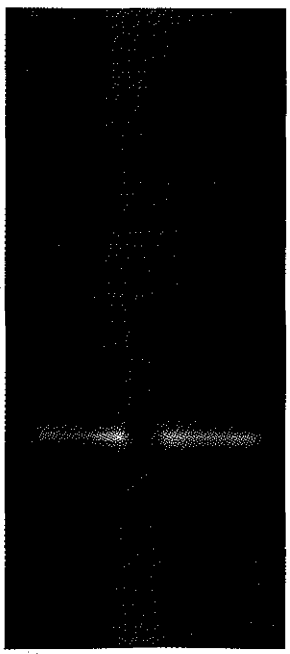
Jatamansi



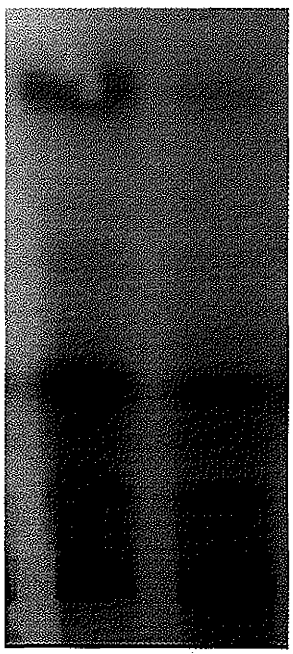
IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)

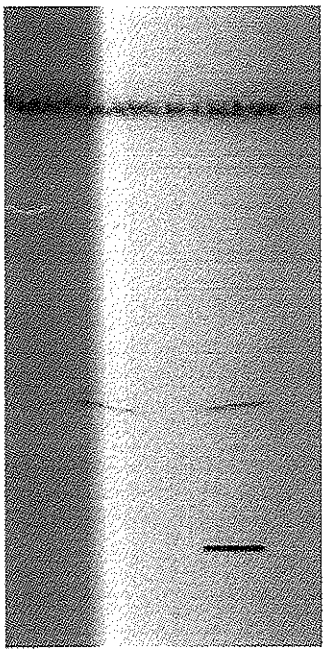


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

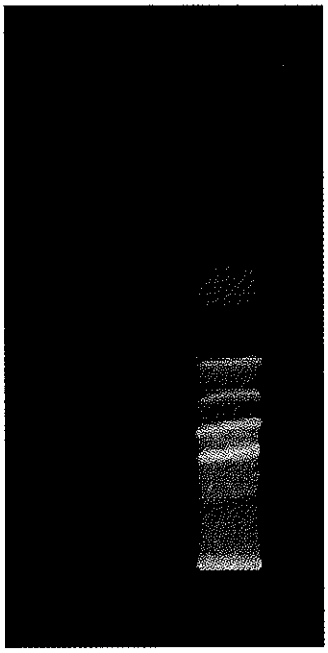
TLC Chromatogram of Jatamansi

AUTHORITY COPY

Kalmegh Dry Extract



IPRS TEST
(Under UV light at 254 nm)

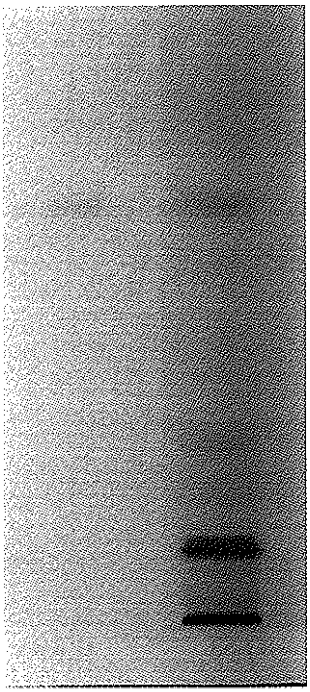


IPRS TEST
(Under UV light at 366 nm)

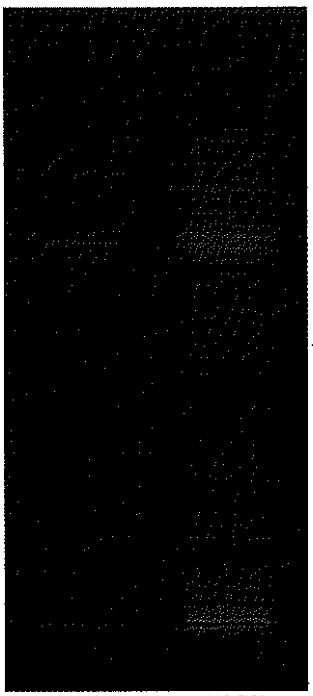
IPRS TEST
(Under day light after spraying with 20 per cent, v/v methanolic sulphuric acid reagent)

TLC Chromatogram of Kalmegh Dry Extract

Karipatta



IPRS TEST
(Under UV light at 254 nm)

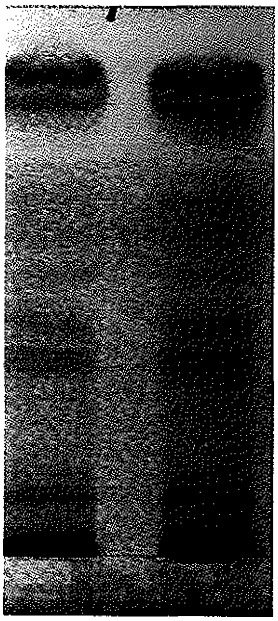


IPRS TEST
(Under UV light at 366 nm)

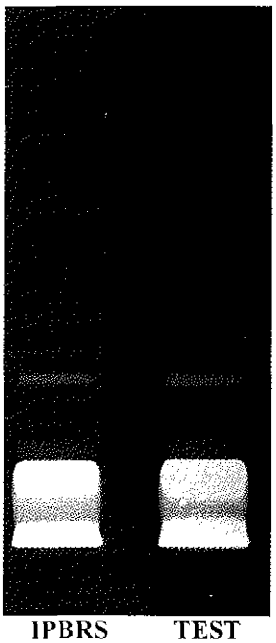
TLC Chromatogram of Karipatta

UNCONTROLLED COPY

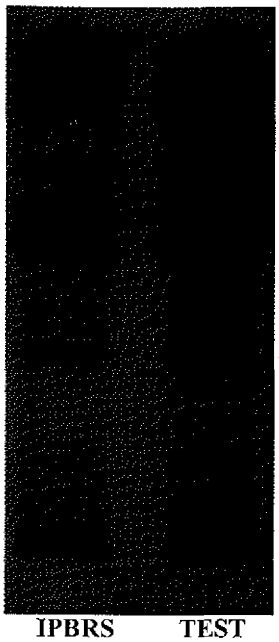
Kasni



IPBRS TEST
(Under UV light at 254 nm)



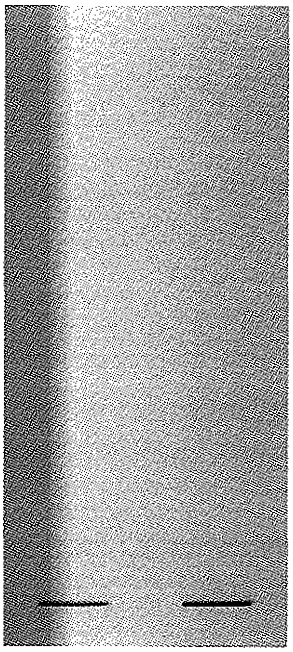
IPBRS TEST
(Under UV light at 366 nm)



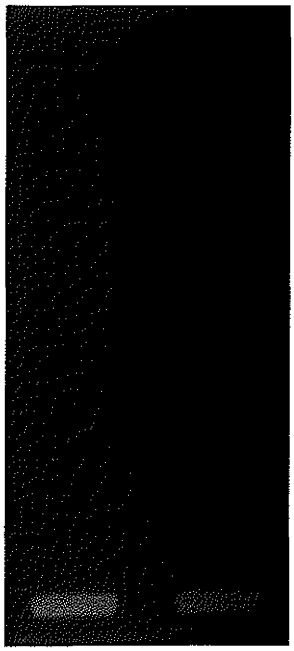
IPBRS TEST
(After spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Kasni

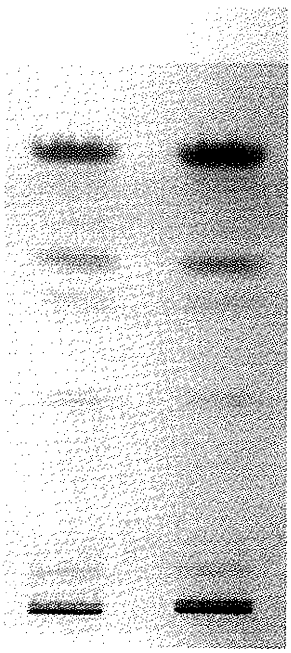
Kaunch



IPBRS TEST
(Under UV light at 254 nm)



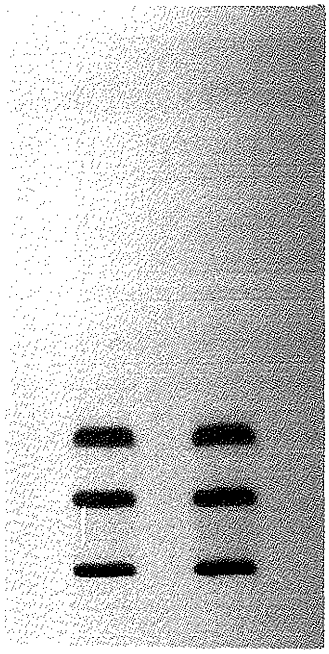
IPBRS TEST
(Under UV light at 366 nm)



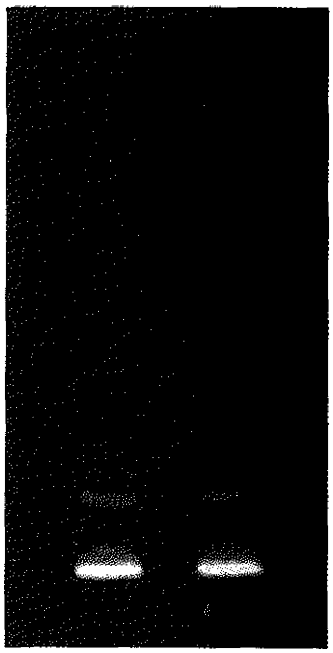
IPBRS TEST
(After spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Kaunch

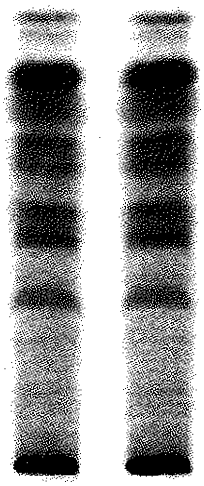
Kunduru



IPBRS TEST
(Under UV light at 254 nm)



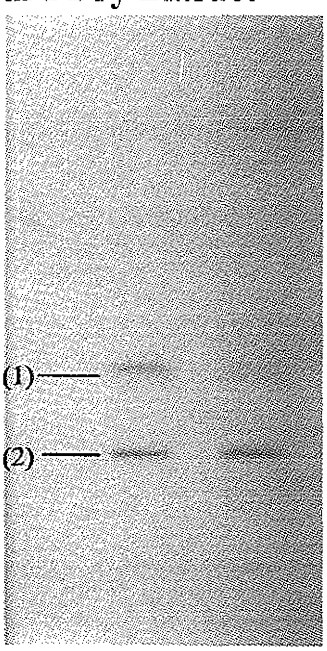
IPBRS TEST
(Under UV light at 366 nm)



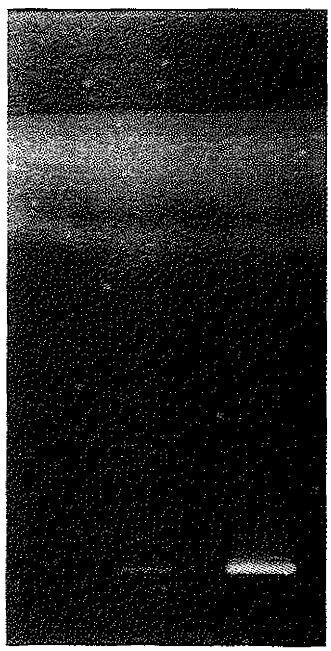
IPBRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

TLC Chromatogram of Kunduru

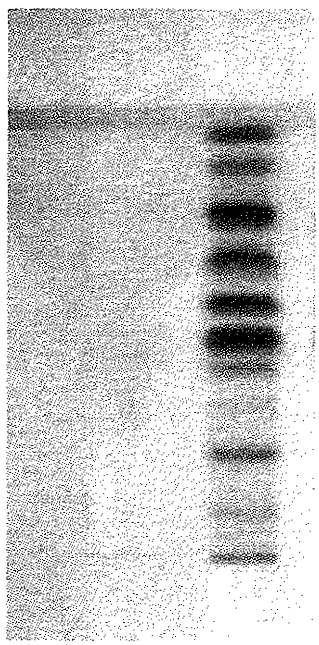
Kunduru Dry Extract



IPRS TEST
(Under UV light at 254 nm)



IPRS TEST
(Under UV light at 366 nm)

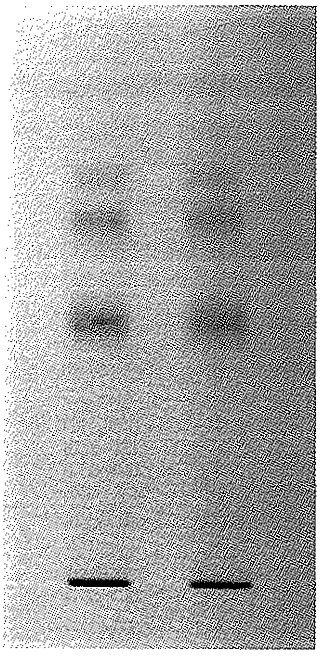


IPRS TEST
(Under day light after spraying with 10 per cent, v/v methanolic sulphuric acid reagent)

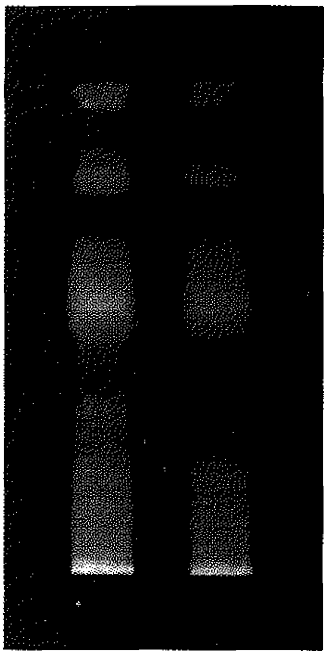
(IPRS1 = 11-keto-β-boswellic acid IPRS and IPRS2 = acetyl-11-keto-β-boswellic acid IPRS)

TLC Chromatogram of Kunduru Dry Extract

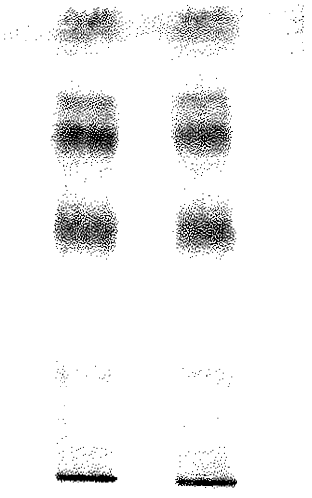
Kutki



IPBRS TEST
(Under UV light at 254 nm)



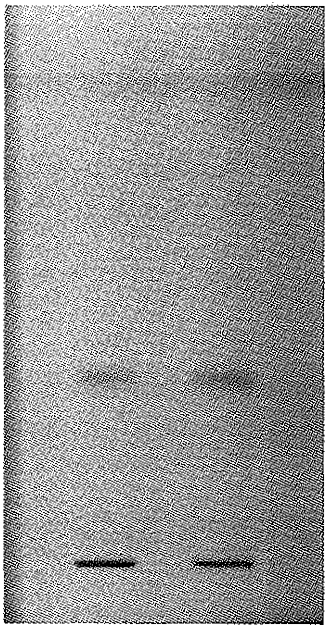
IPBRS TEST
(Under UV light at 366 nm)



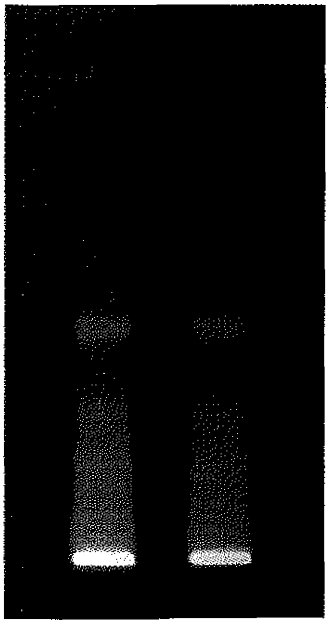
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Kutki

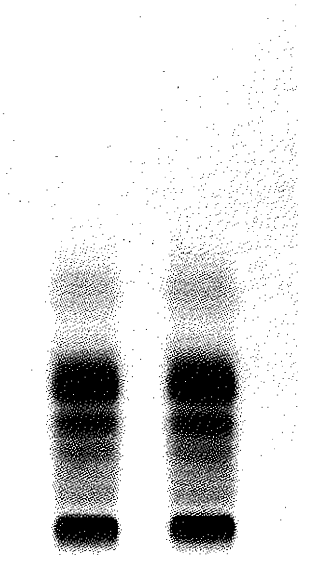
Lasuna



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

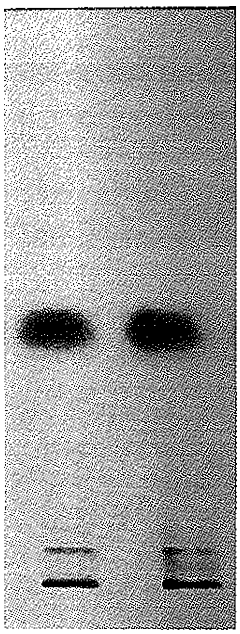


IPBRS TEST
(Under day light after spraying with 0.2 per cent, w/v ninhydrin solution)

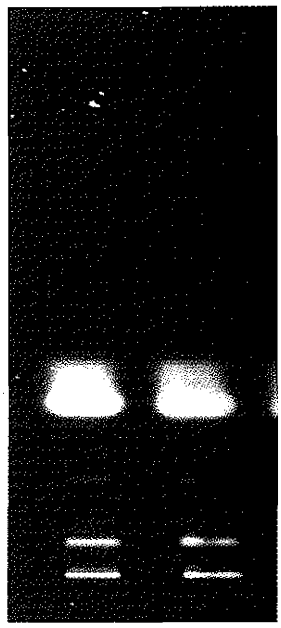
TLC Chromatogram of Lasuna

UNREVISED

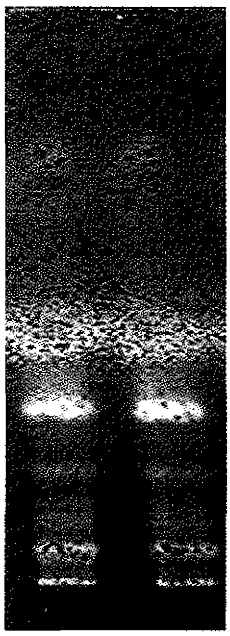
Lavang



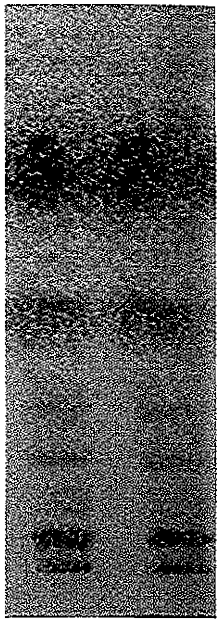
IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)



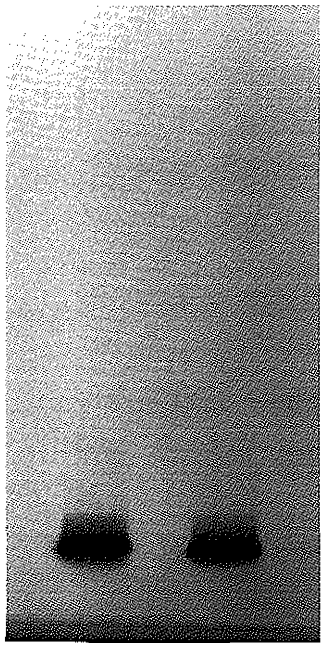
IPBRS TEST
(Under UV light at 366 nm after spraying with vanillin sulphuric acid solution)



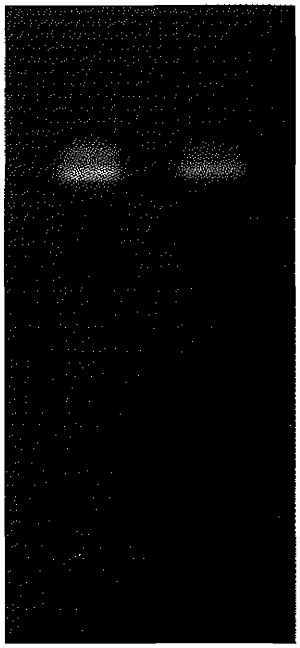
IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid solution)

TLC Chromatogram of Lavang

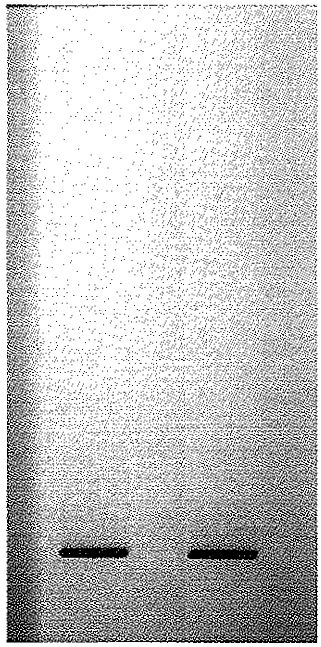
Lodhra



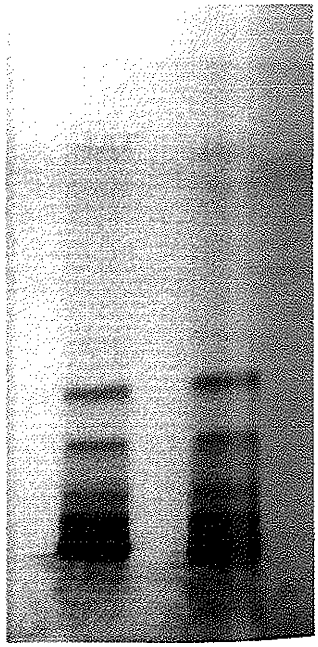
IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under day light)

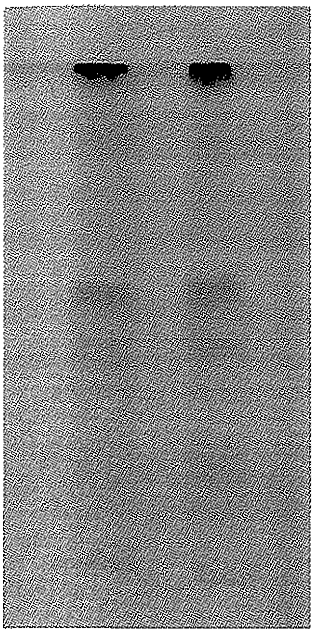


IPBRS TEST
(After spraying with anisaldehyde sulphuric acid reagent)

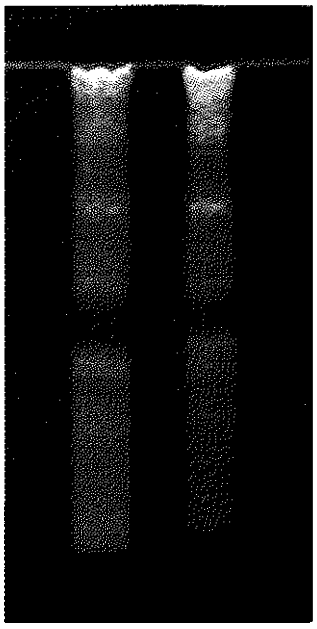
TLC Chromatogram of Lodhra

AUTHORISED COPY

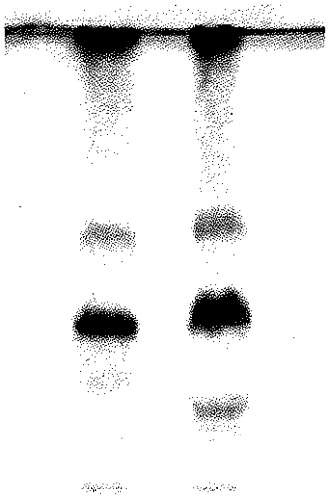
Mandukaparni



IPBRS TEST
(Under UV light at 254 nm)



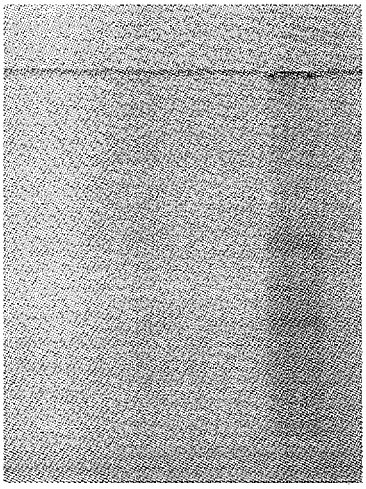
IPBRS TEST
(Under UV light at 366 nm)



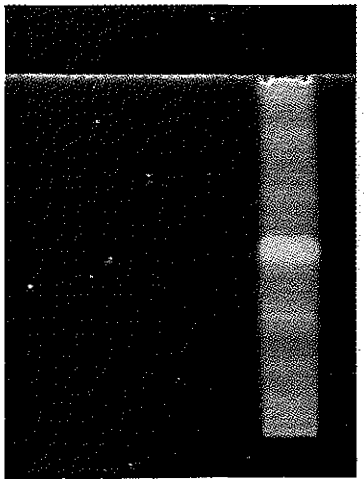
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Mandukaparni

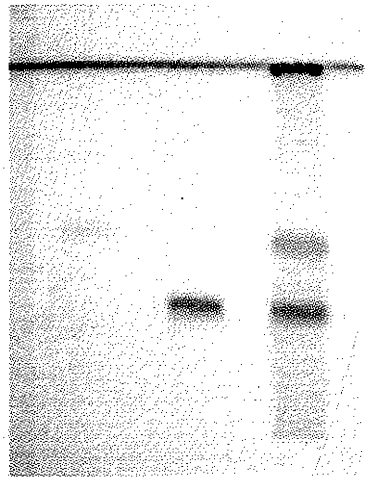
Mandukaparni Dry Extract



IPRS1 IPRS2 TEST
(Under UV light at 254 nm)



IPRS1 IPRS2 TEST
(Under UV light at 366 nm)



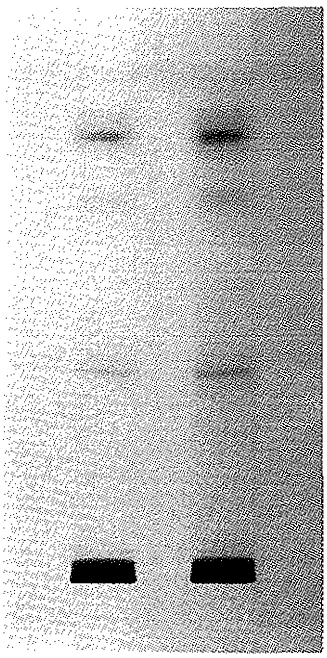
IPRS1 IPRS2 TEST
(Under day light after spraying with 10 per cent methanolic sulphuric acid)

(IPRS1 = Asiaticoside IPRS and IPRS2 = Madecassoside IPRS)

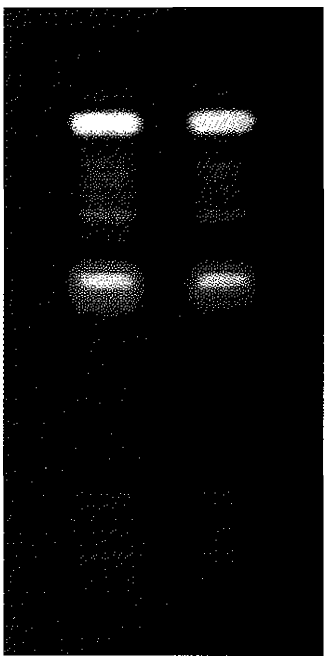
TLC Chromatogram of Mandukaparni Dry Extract

UNAUTHORISED COPY

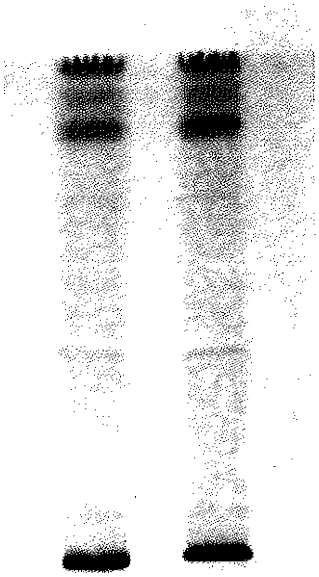
Manjistha



IPBRS TEST
(Under UV light at 254 nm)



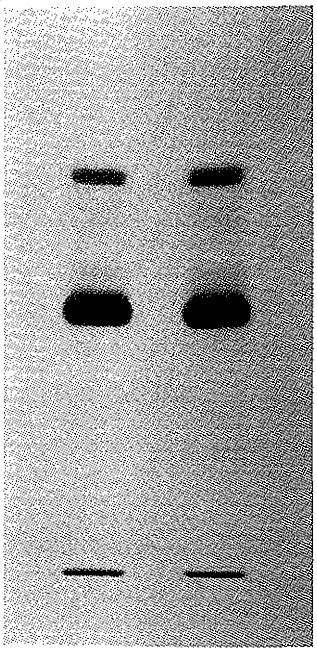
IPBRS TEST
(Under UV light at 366 nm)



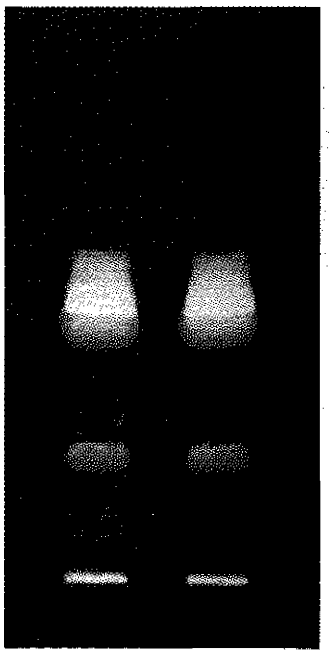
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Manjishta

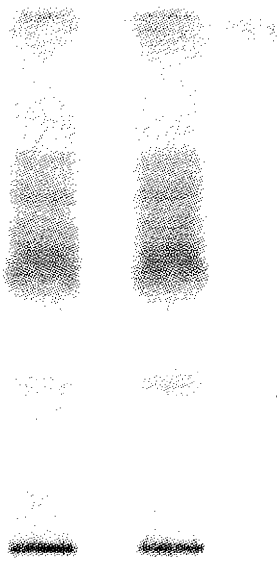
Maricha



IPBRS TEST
(Under UV light at 254 nm)



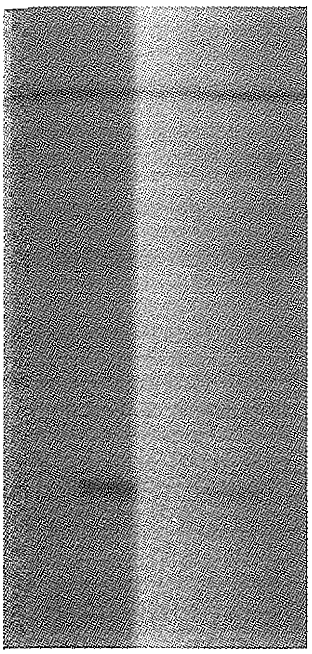
IPBRS TEST
(Under UV light at 366 nm)



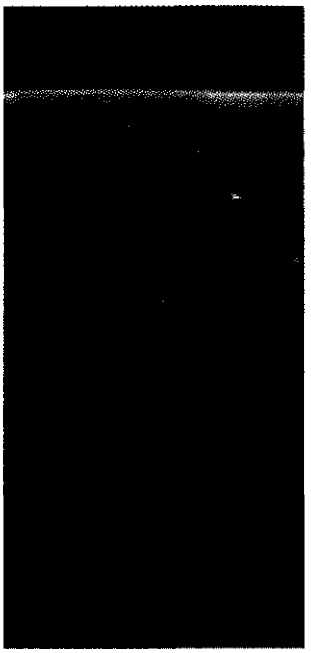
IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid reagent)

TLC Chromatogram of Maricha

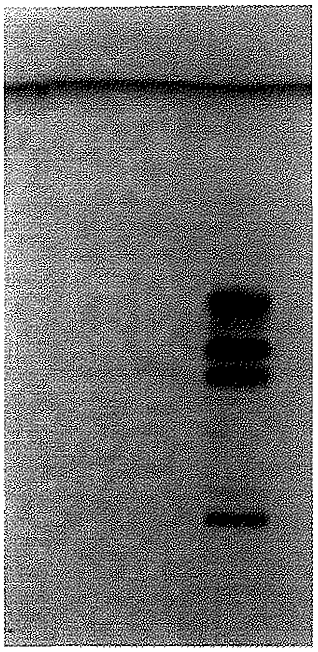
Methi



IPRS TEST
(Under UV light at 254 nm)



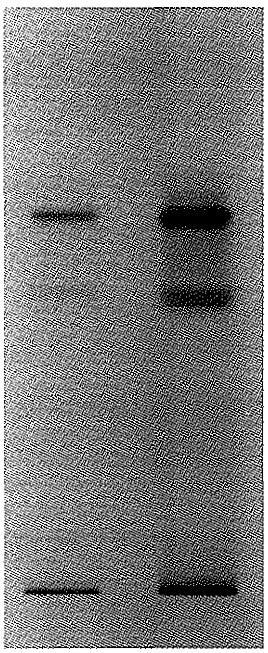
IPRS TEST
(Under UV light at 366 nm)



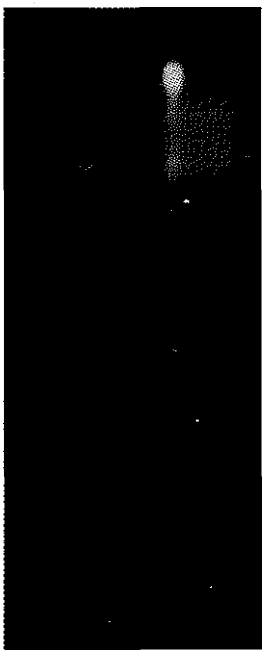
IPRS TEST
(Under day light after spraying with potassium iodobismuthate solution)

TLC Chromatogram of Methi

Milk Thistle



IPBRS TEST
(Under UV light at 254 nm)

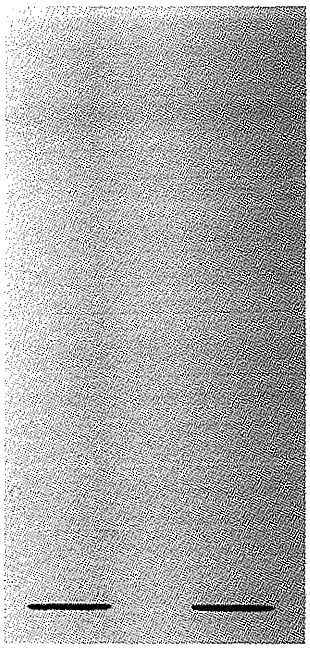


IPBRS TEST
(Under UV light at 366 nm)

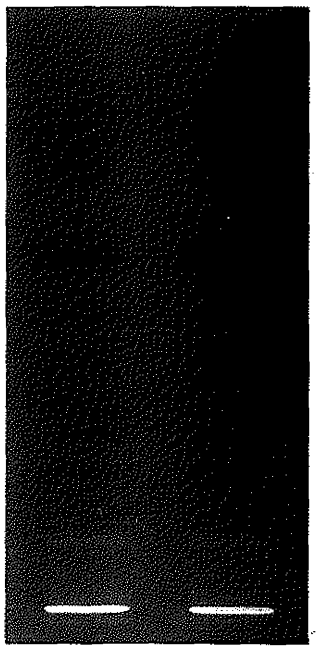
TLC Chromatogram of Milk Thistle

UNAUTHORISED COPY

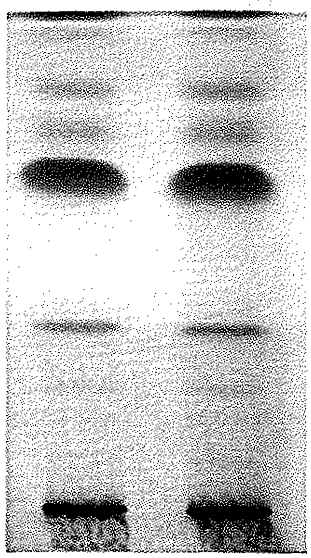
Mirch



IPBRS TEST
(Under UV light at 254 nm)



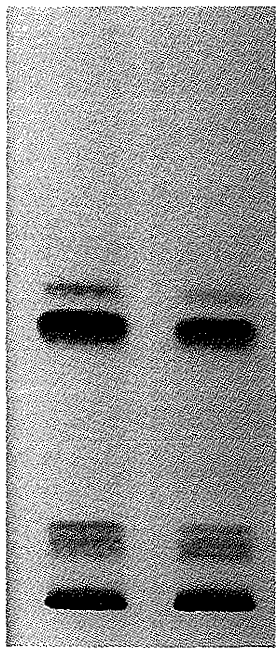
IPBRS TEST
(Under UV light at 366 nm)



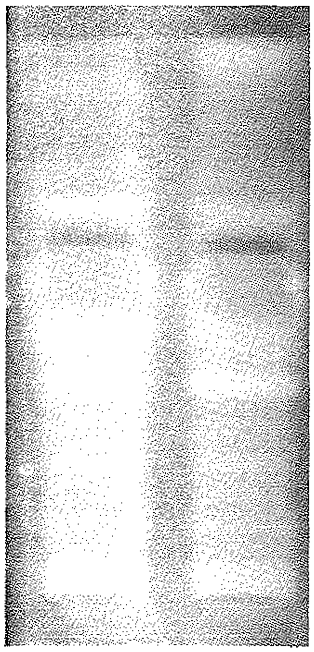
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Mirch

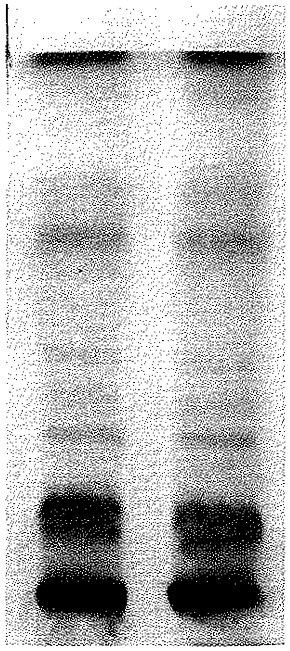
Nagakesar



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

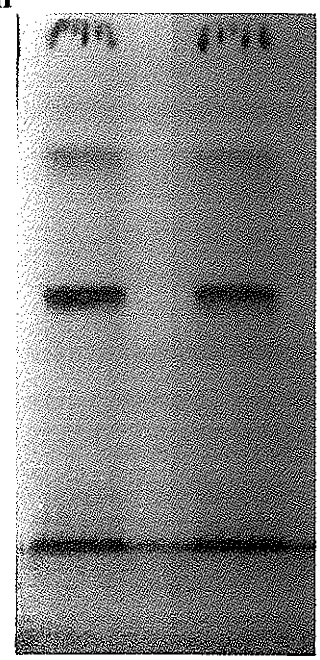


IPBRS TEST
(Under day light after spraying with anisaldehyde reagent)

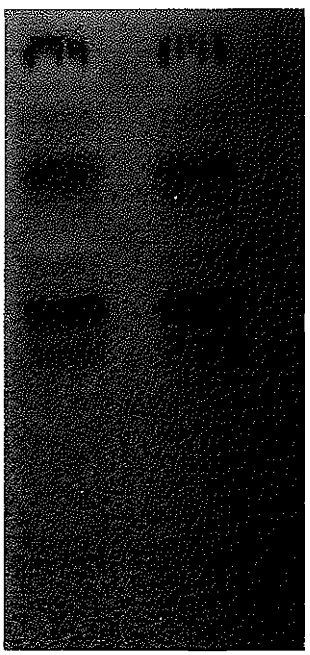
TLC Chromatogram of Nagakesar

UNAUTHORISED COPY

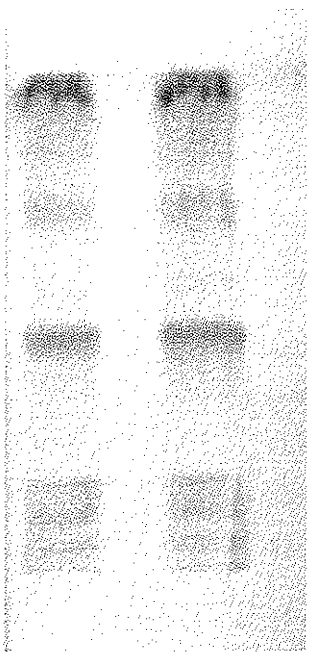
Neem



IPBRS TEST
(Under UV light at 254 nm)



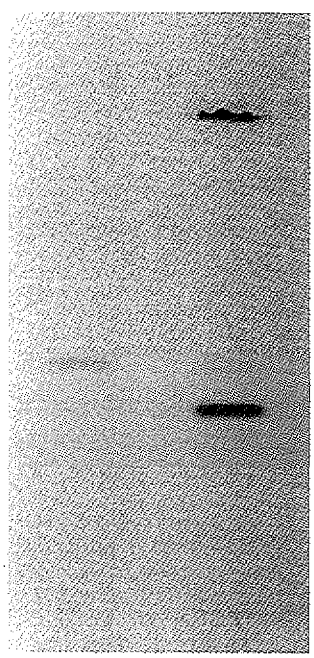
IPBRS TEST
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric acid reagent)



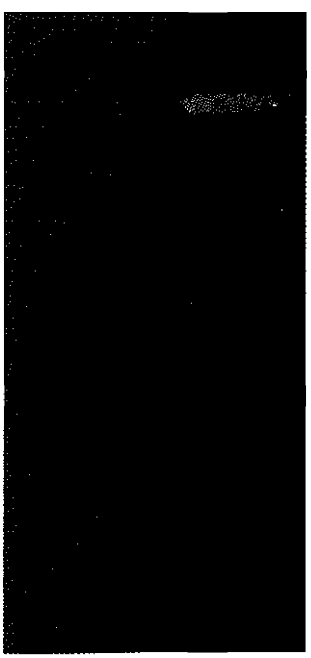
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Neem

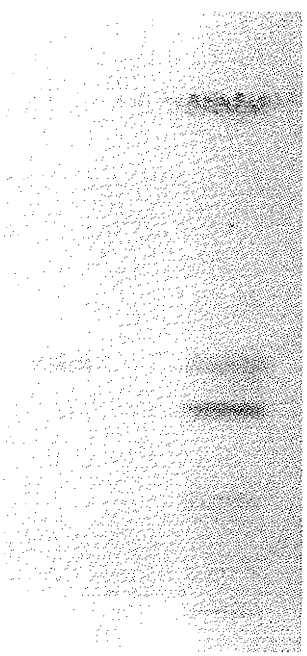
Nirgundi



IPBRS TEST
(Under UV light at 254 nm)



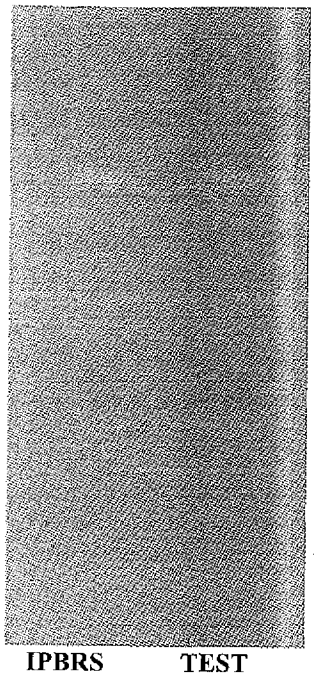
IPBRS TEST
(Under UV light at 366 nm)



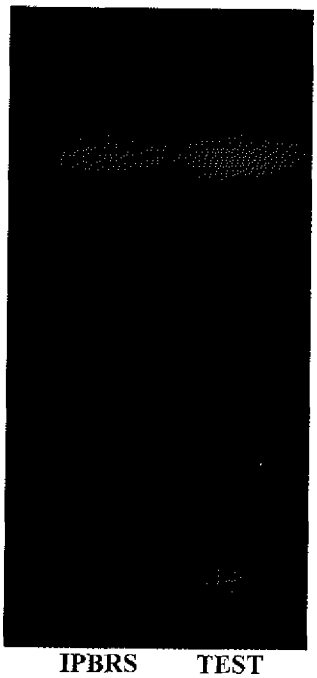
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Nirgundi

Noni



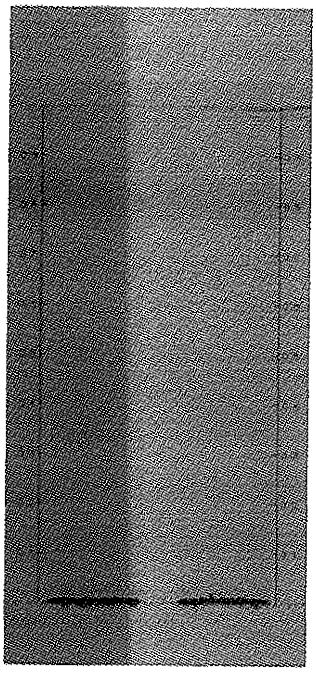
IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

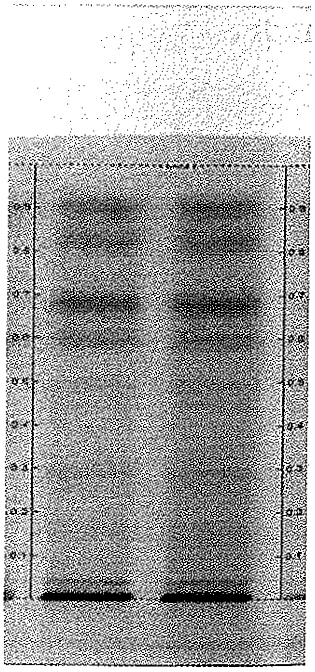
TLC Chromatogram of Noni

Patha



IPBRS TEST
(Under UV light at 254 nm)

IPBRS TEST
(Under UV light at 366 nm)

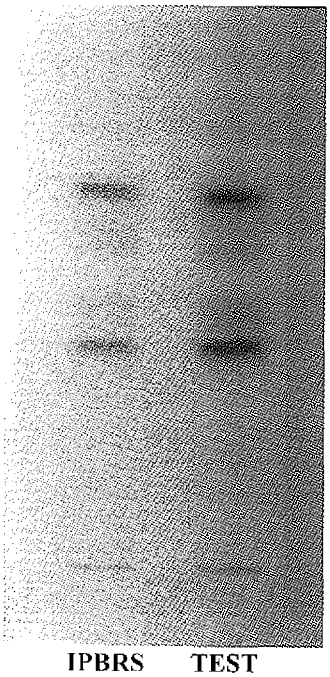


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

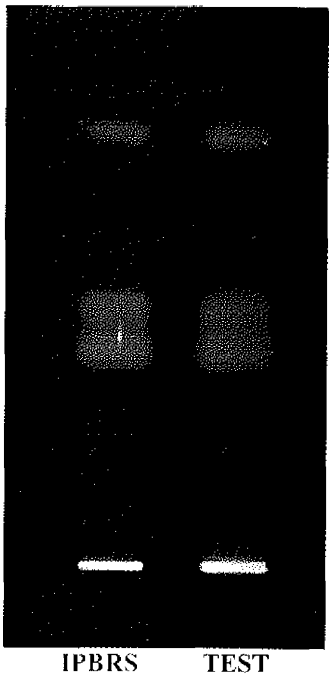
TLC Chromatogram of Patha

UNAUTHORISED COPY

Pippali (Large)



IPBRS TEST
(Under UV light at 254 nm)

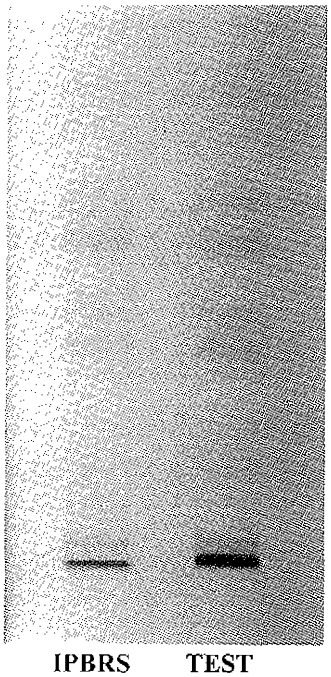


IPBRS TEST
(Under UV light at 366 nm)

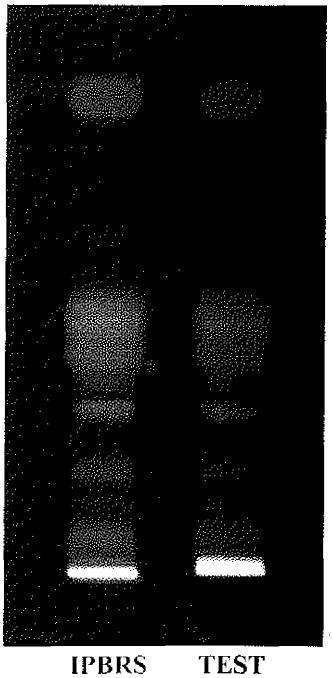
IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid reagent)

TLC Chromatogram of Pippali (Large)

Pippali (Small)



IPBRS TEST
(Under UV light at 254 nm)



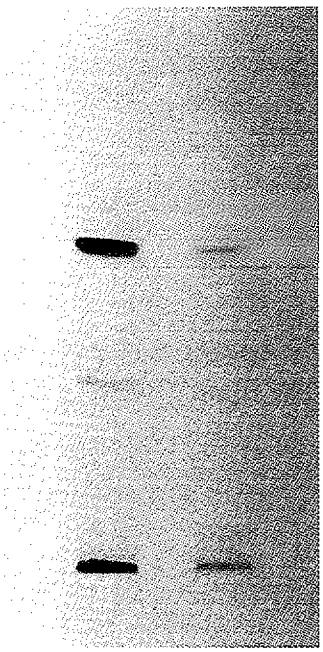
IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid reagent)

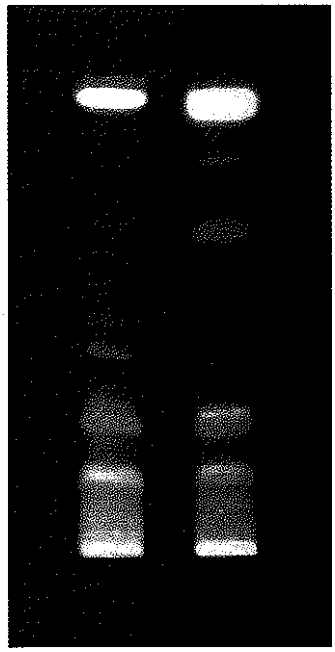
TLC Chromatogram of Pippali (Small)

UNCONTROLLED

Punarnava



IPBRS TEST
(Under UV light at 254 nm)

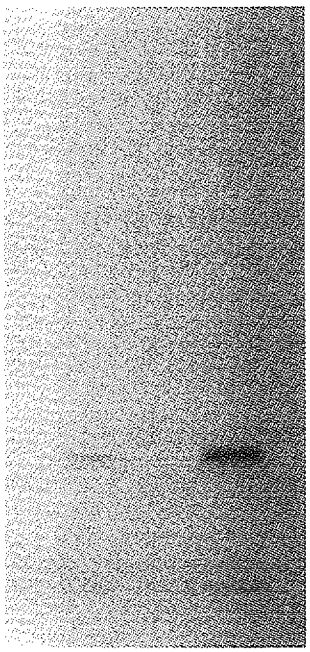


IPBRS TEST
(Under UV light at 366 nm)

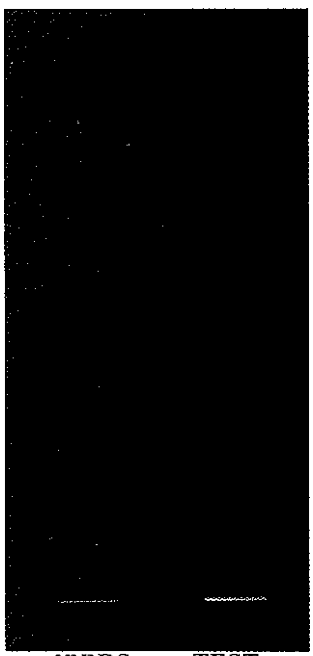
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Punarnava

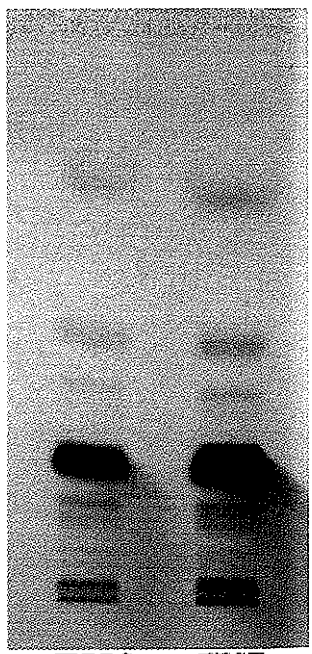
Puskara



IPBRS TEST
(Under UV light at 254 nm)



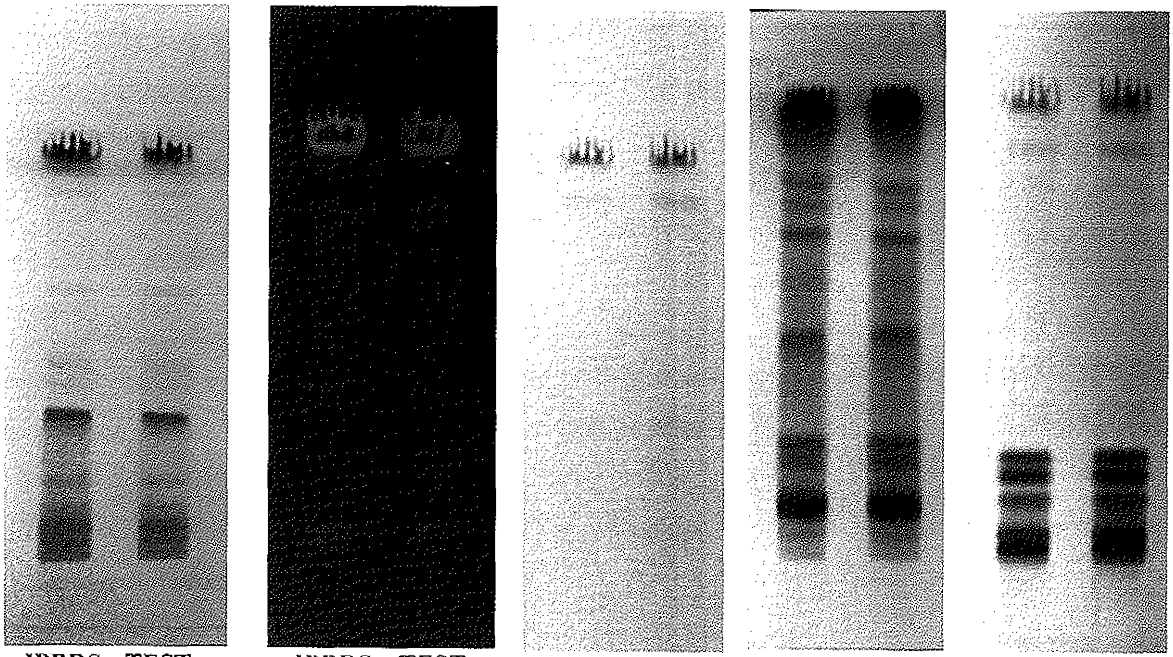
IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Puskara

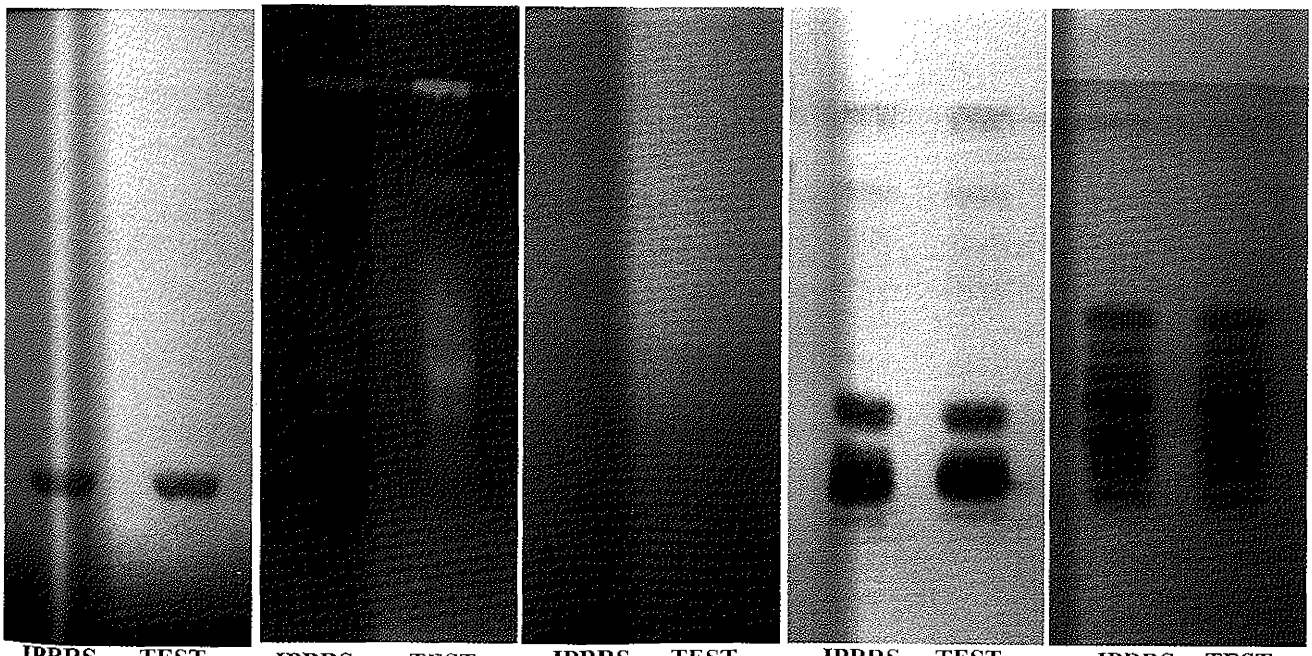
Sahajana Leaf



IPBRS TEST (Under UV light at 254 nm) | IPBRS TEST (Under UV light at 366 nm) | IPBRS TEST (Under day light) | IPBRS TEST (Under day light after spraying with 5.0 per cent sulphuric acid) | IPBRS TEST (Under day light after spraying with 0.5 per cent ninhydrin solution)

TLC Chromatogram of Sahajana Leaf

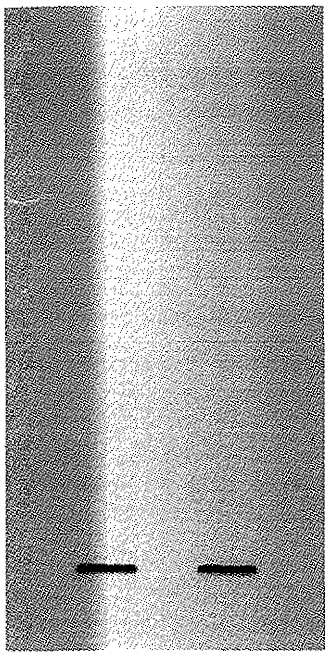
Sahajana Stick



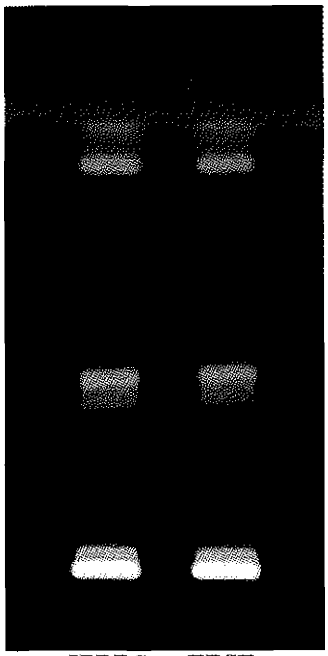
IPBRS TEST (Under UV light at 254 nm) | IPBRS TEST (Under UV light at 366 nm) | IPBRS TEST (Under day light) | IPBRS TEST (Under day light after spraying with 5.0 per cent sulphuric acid) | IPBRS TEST (Under day light after spraying with 0.5 per cent ninhydrin solution)

TLC Chromatogram of Sahajana Stick

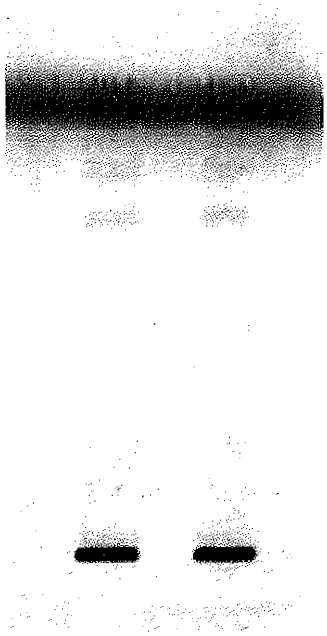
Sarpagandha



IPBRS TEST
(Under UV light at 254 nm)



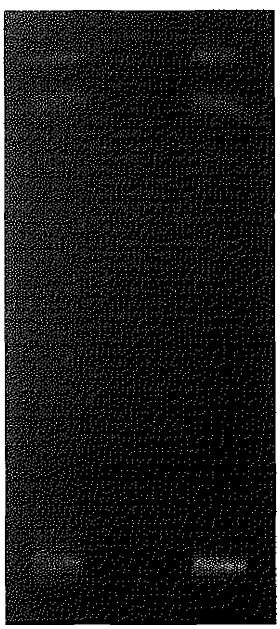
IPBRS TEST
(Under UV light at 366 nm)



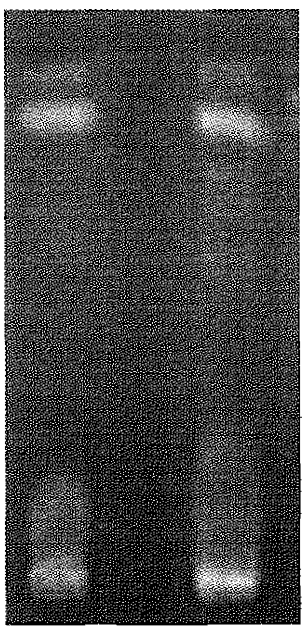
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Sarpagandha

Sarpagandha Powder



IPBRS TEST
(Under UV light at 366 nm)

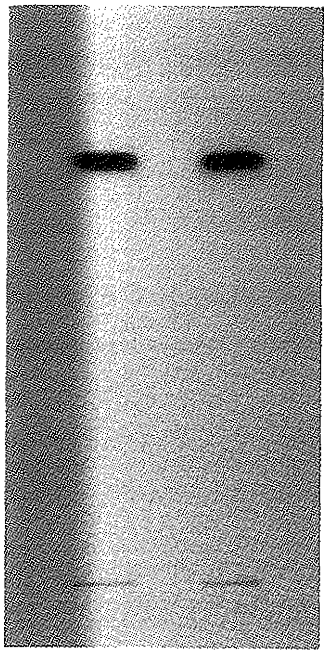


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

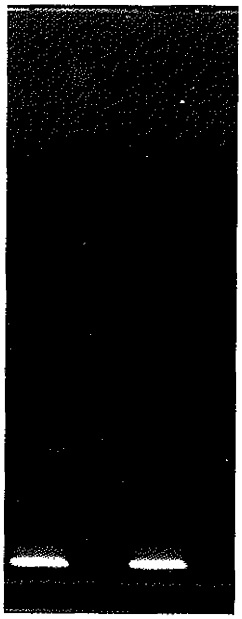
TLC Chromatogram of Sarpagandha Powder

UNAUTHORISED COPY

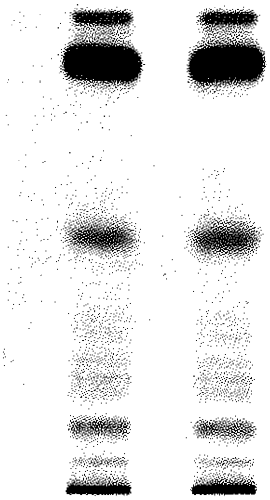
Saunf



IPBRS TEST
(Under UV light at 254 nm)



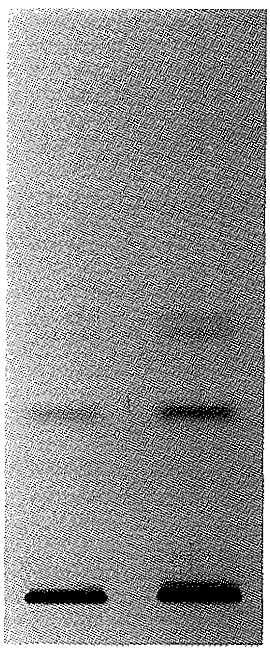
IPBRS TEST
(Under UV light at 366 nm)



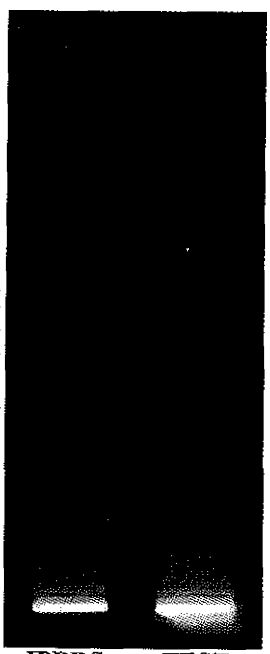
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Saunf

Schisandra Fruit



IPBRS TEST
(Under UV light at 254 nm)

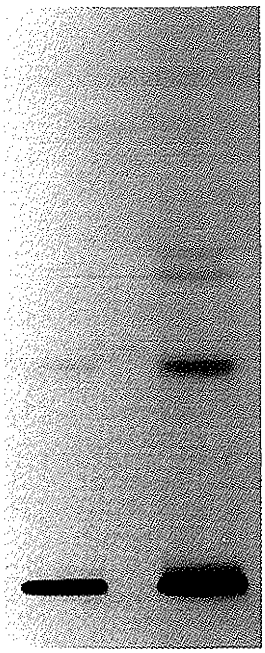


IPBRS TEST
(Under UV light at 366 nm)

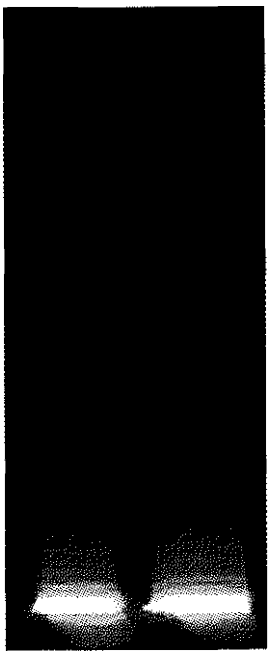
TLC Chromatogram of Schisandra Fruit

UNAUTHORISED COPY

Schisandra Dry Extract



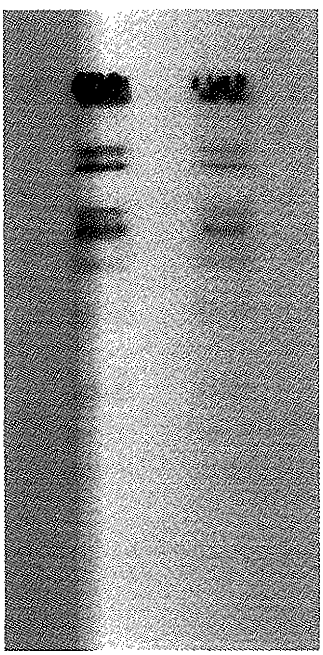
IPBRS TEST
(Under UV light at 254 nm)



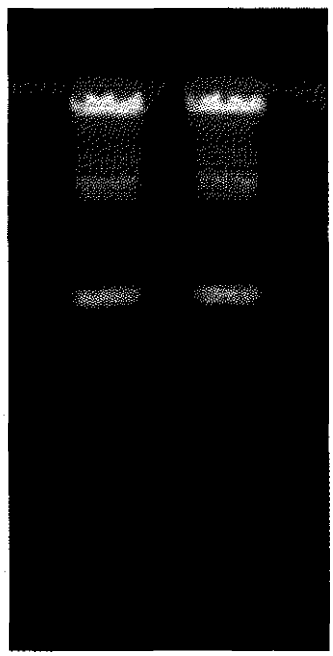
IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Schisandra Dry Extract

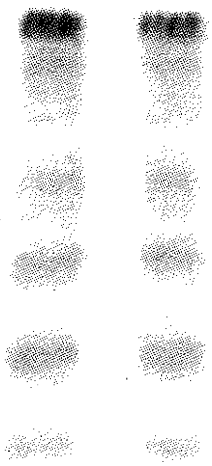
Senna Leaf



IPBRS TEST
(Under UV light at 254 nm)



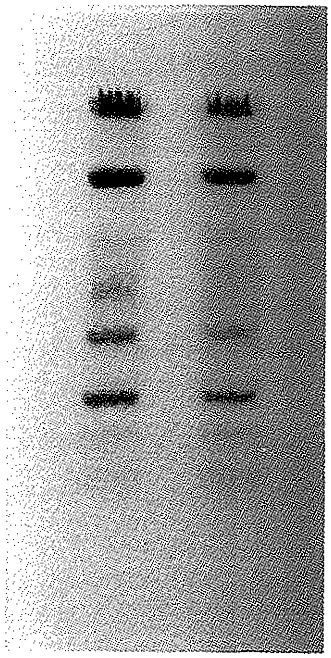
IPBRS TEST
(Under UV light at 366 nm)



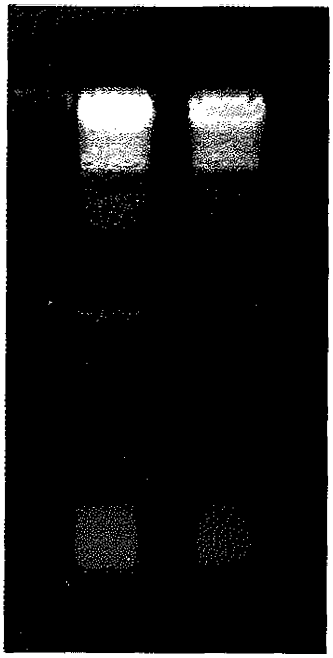
IPBRS TEST
(Under day light after spraying with 20 per cent, v/v nitric acid solution)

TLC Chromatogram of Senna Leaf

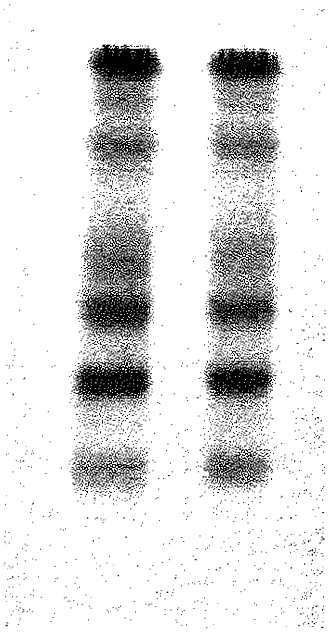
Senna Pods



IPBRS TEST
(Under UV light at 254 nm)



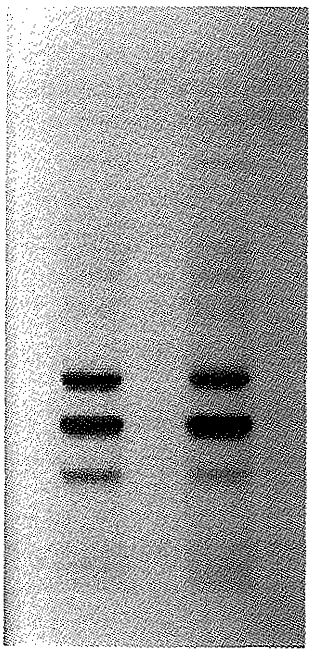
IPBRS TEST
(Under UV light at 366 nm)



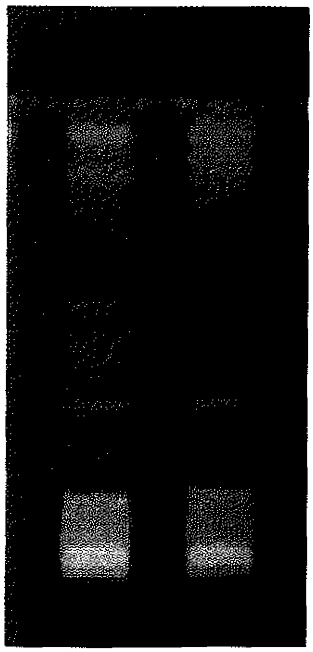
IPBRS TEST
(Under day light after spraying with 20 per cent, v/v nitric acid solution)

TLC Chromatogram of Senna Pods

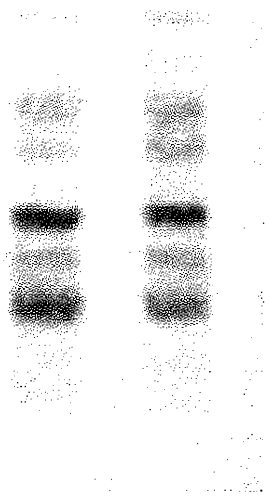
Senna Dry Extract



IPBRS TEST
(Under UV light at 254 nm)



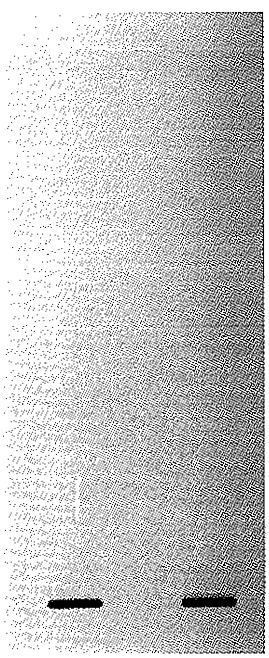
IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under day light after spraying with 20 per cent, v/v nitric acid solution)

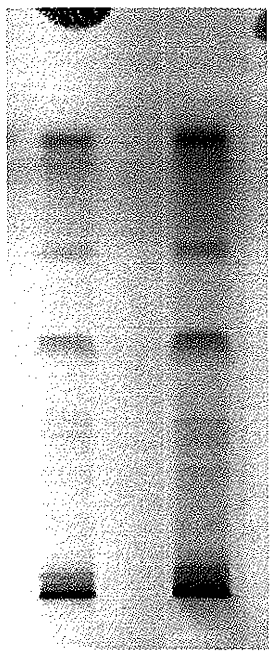
TLC Chromatogram of Senna Dry Extract

Shankhpushpi

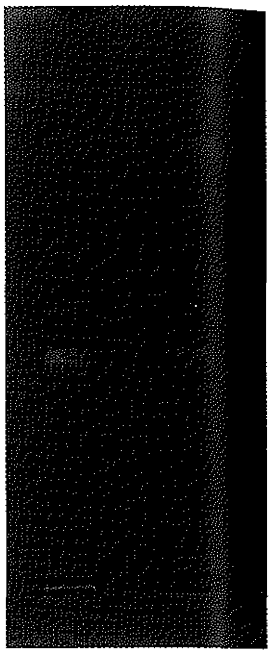


IPBRS TEST
(Under UV light at 254 nm)

IPBRS TEST
(Under UV light at 366 nm)



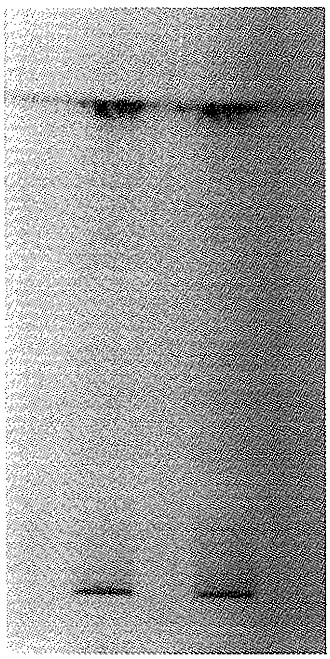
IPBRS TEST
(After spraying with anisaldehyde sulphuric acid reagent)



IPBRS TEST
(At 366 nm after spraying with anisaldehyde sulphuric acid reagent)

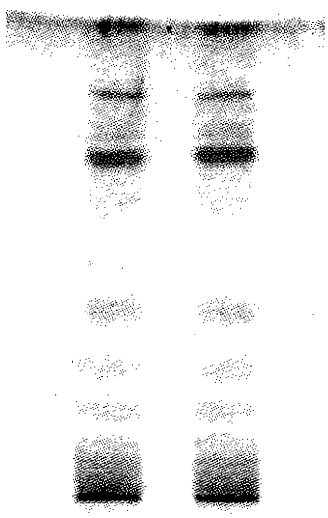
TLC Chromatogram of Shankhpushpi

Shatavari



IPBRS TEST
(Under UV light at 254 nm)

IPBRS TEST
(Under UV light at 366 nm)

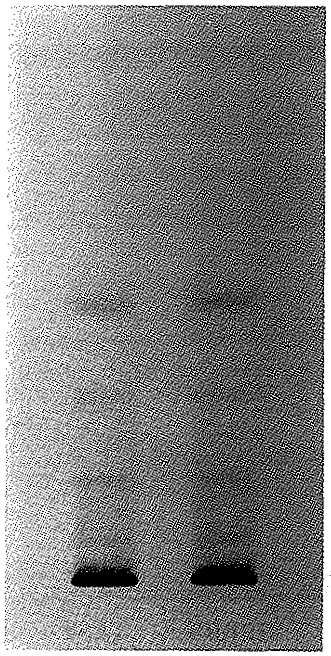


IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid reagent)

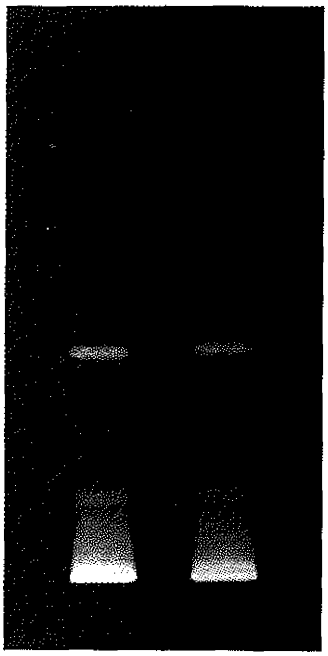
TLC Chromatogram of Shatavari

COPY

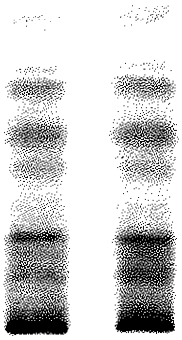
Shati



IPBRS TEST
(Under UV light at 254 nm)



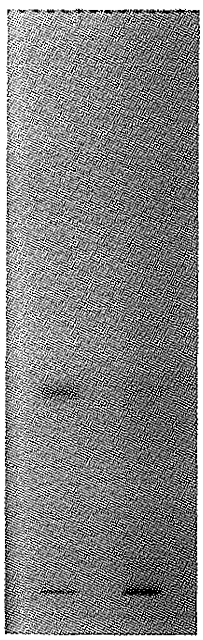
IPBRS TEST
(Under UV light at 366 nm)



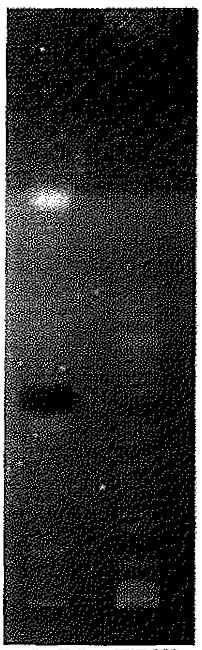
IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Shati

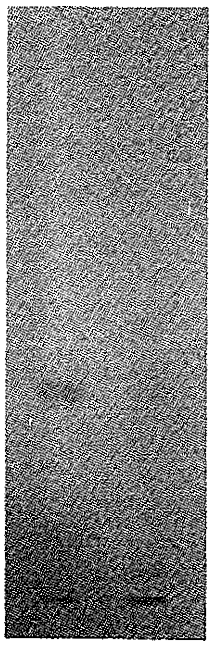
Siris



IPRS TEST
(Under UV light at 254 nm)



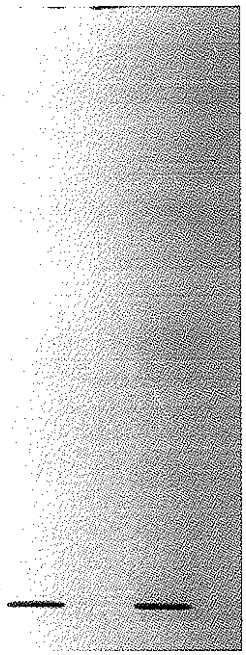
IPRS TEST
(Under UV light at 366 nm)



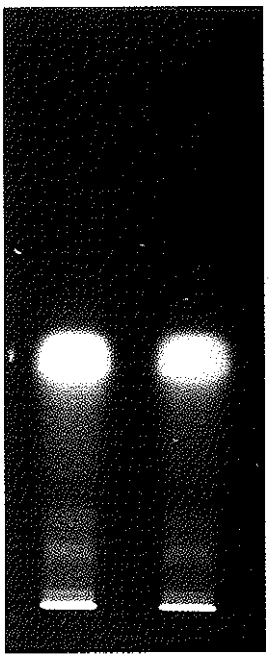
IPRS TEST
(Under day light)

TLC Chromatogram of Siris

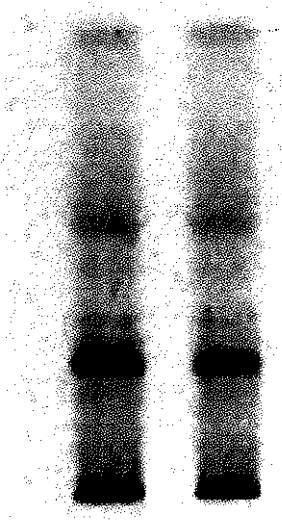
Sunthi



IPBRS TEST
(Under UV light at 254 nm)



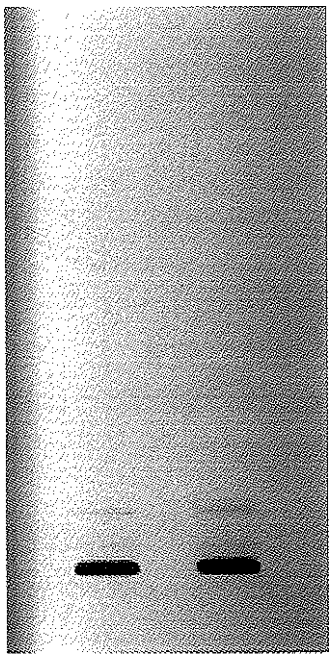
IPBRS TEST
(Under UV light at 366 nm)



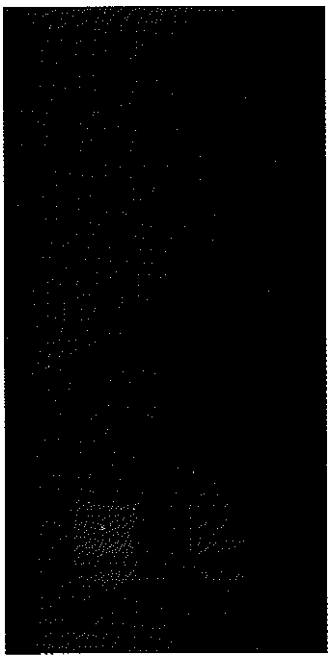
IPBRS TEST
(Under day light after spraying with vanillin sulphuric acid reagent)

TLC Chromatogram of **Sunthi**

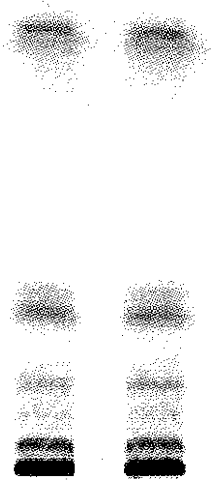
Tulasi



IPBRS TEST
(Under UV light at 254 nm)



IPBRS TEST
(Under UV light at 366 nm)

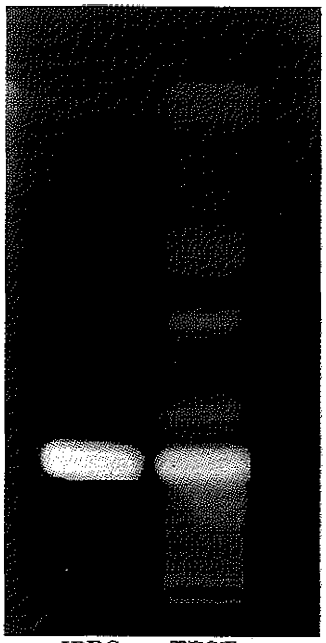


IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of **Tulasi**

UNCORRECTED

Tulasi Dry Extract

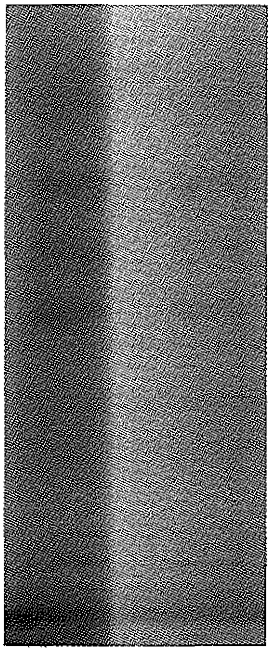


IPRS TEST
(Under UV light at 366 nm after spraying with anisaldehyde sulphuric reagent)

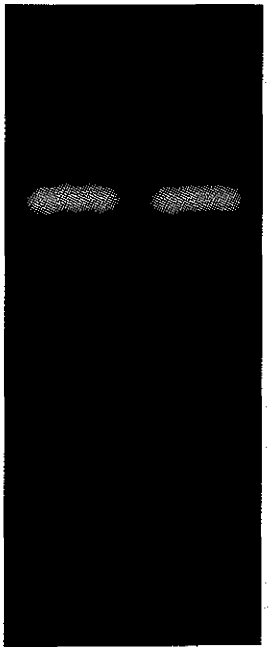
IPRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Tulasi Dry Extract

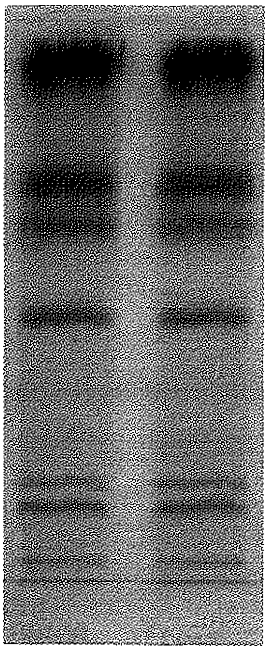
Tvak



IPBRS TEST
(Under UV light at 254 nm)



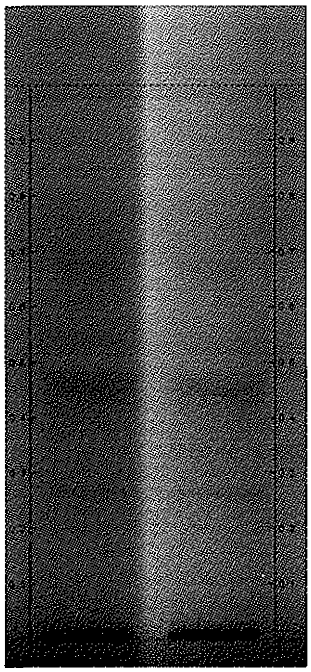
IPBRS TEST
(Under UV light at 366 nm)



IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

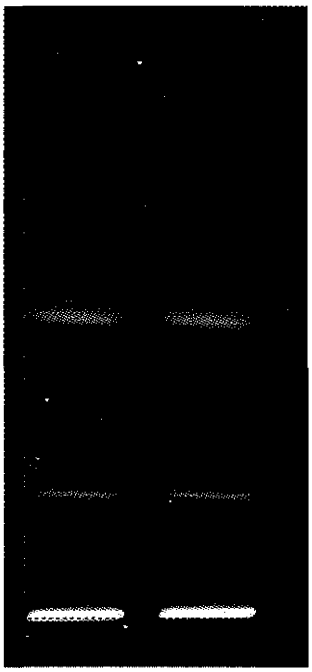
TLC Chromatogram of Tvak

Upakunchika



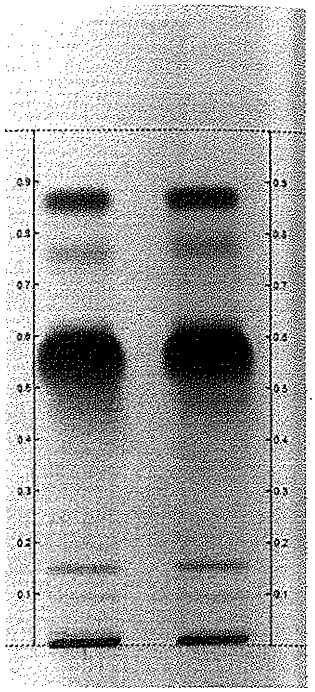
IPBRS TEST

(Under UV light at 254 nm)



IPBRS TEST

(Under UV light at 366 nm)

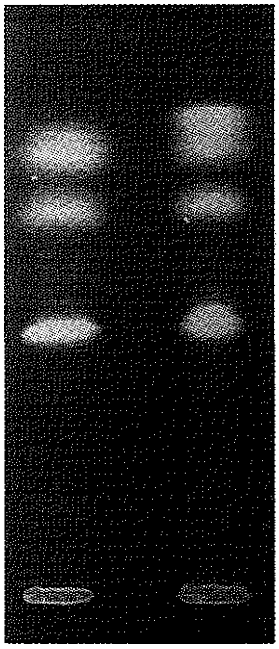


IPBRS TEST

(Under day light after spraying with anisaldehyde sulphuric acid reagent)

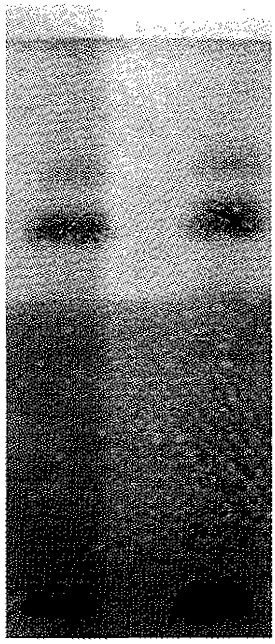
TLC Chromatogram of Upakunchika

Valerian Root



IPBRS TEST

(Under UV light at 366 nm)

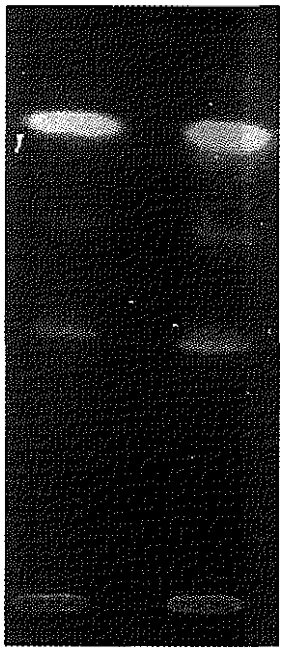


IPBRS TEST

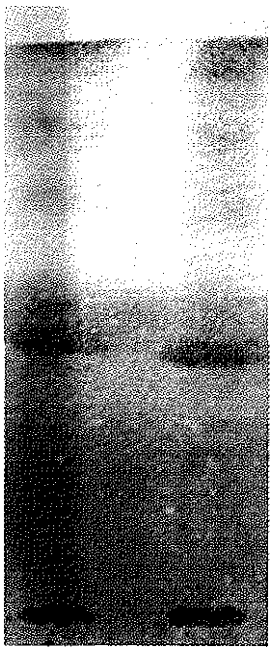
(Under day light after spraying with anisaldehyde reagent)

TLC Chromatogram of Valerian Root

Valerian Dry Extract



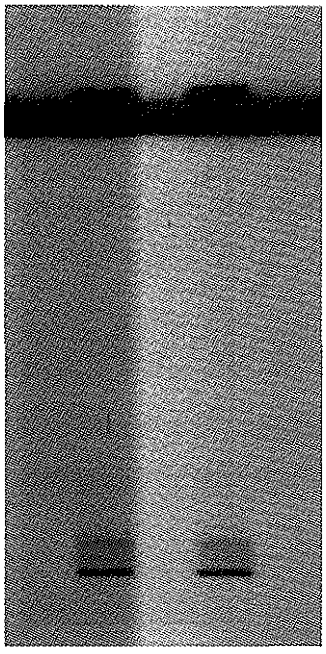
IPBRS TEST
(Under UV light at 366 nm)



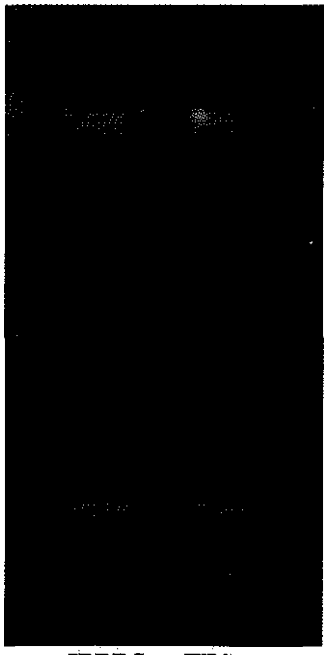
IPBRS TEST
(Under day light after spraying with anisaldehyde reagent)

TLC Chromatogram of Valerian Dry Extract

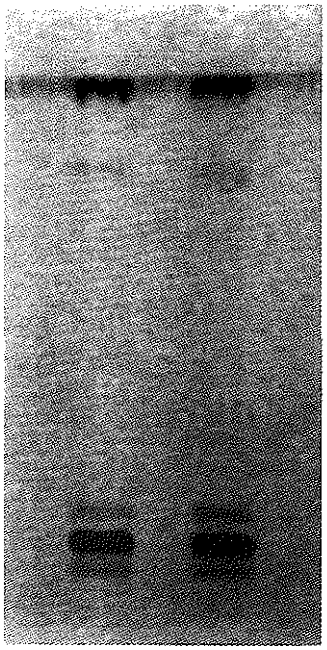
Vasaka



IPBRS TEST
(Under UV light at 254 nm)



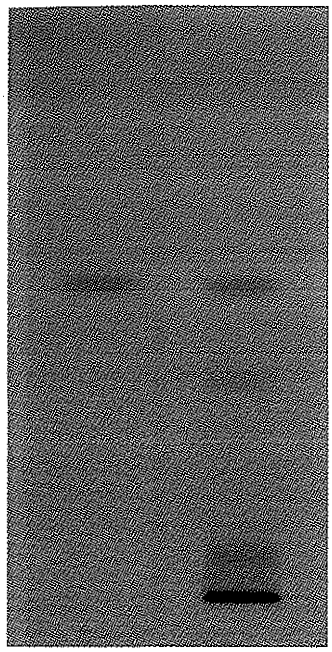
IPBRS TEST
(Under UV light at 366 nm)



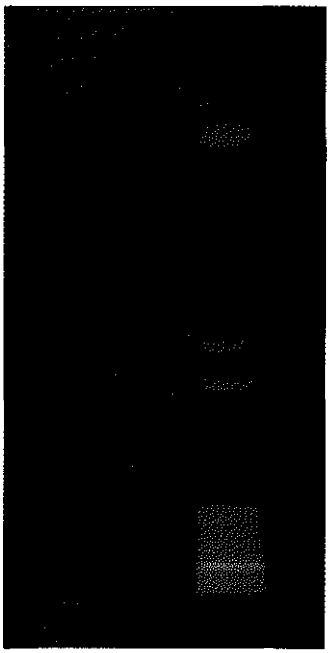
IPBRS TEST
(Under day light after spraying with Dragendorff's reagent)

TLC Chromatogram of Vasaka

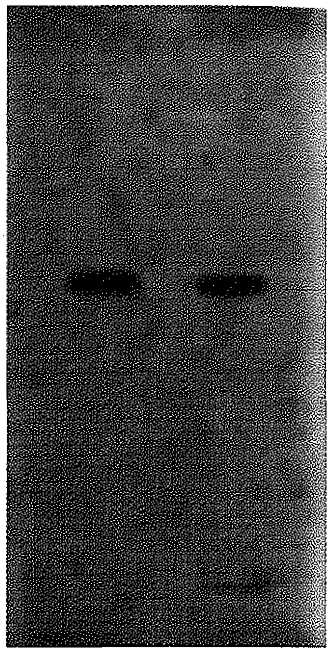
Vasaka Extract



IPRS TEST
(Under UV light at 254 nm)



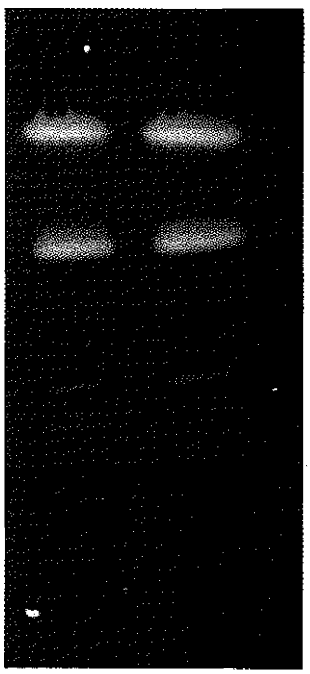
IPRS TEST
(Under UV light at 366 nm)



IPRS TEST
(Under day light after spraying with Dragendorff's reagent)

TLC Chromatogram of Vasaka Extract

Vidanga

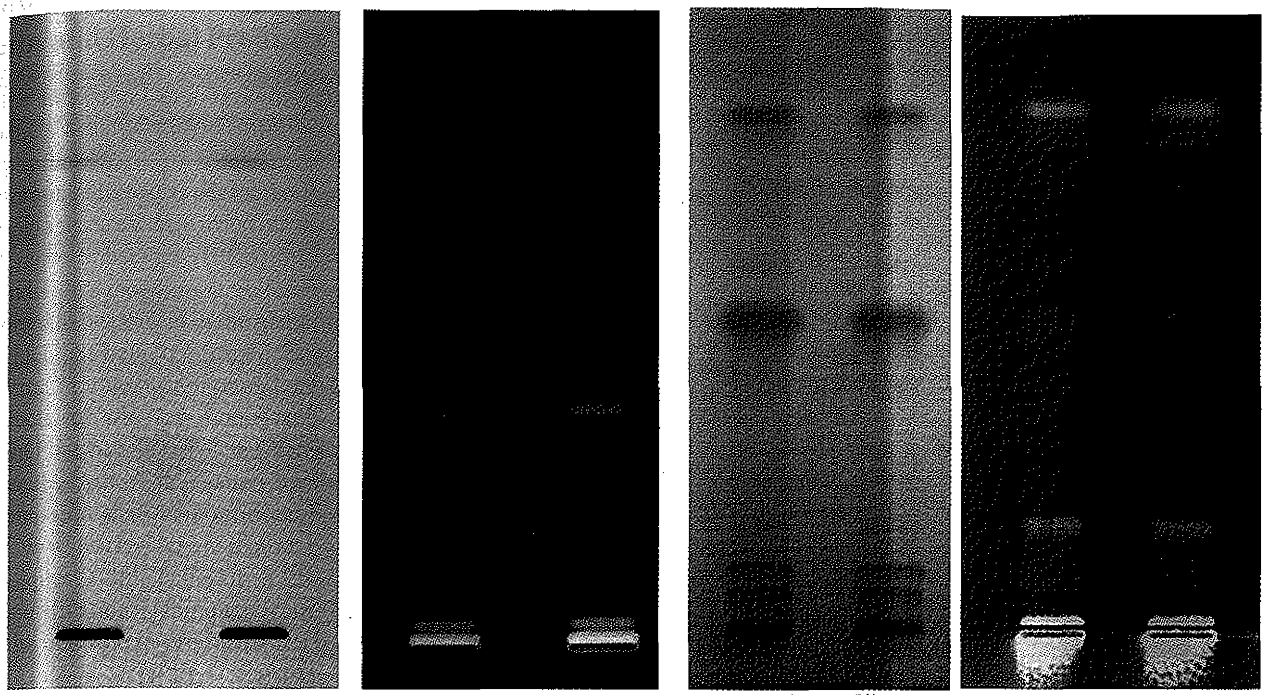


IPBRS TEST
(Under UV light at 366 nm)

TLC Chromatogram of Vidanga

IPR RISED

Vijayasara



IPBRS TEST
(Under UV light at 254 nm)

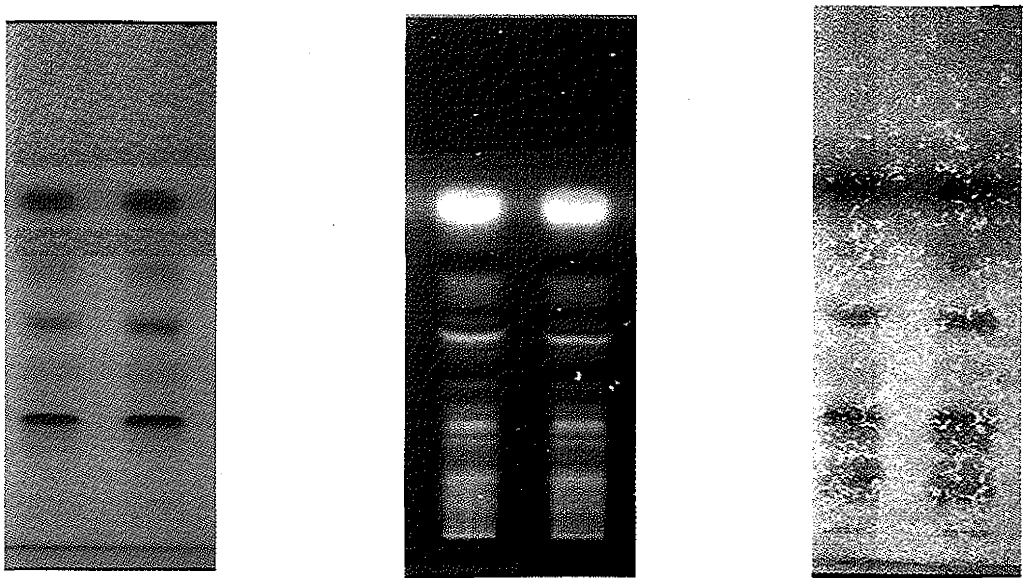
IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light after spraying with liebermann-burchard reagent)

IPBRS TEST
(Under UV light at 366 nm after spraying with liebermann-burchard reagent)

TLC Chromatogram of Vijayasara

Yasti



IPBRS TEST
(Under UV light at 254 nm)

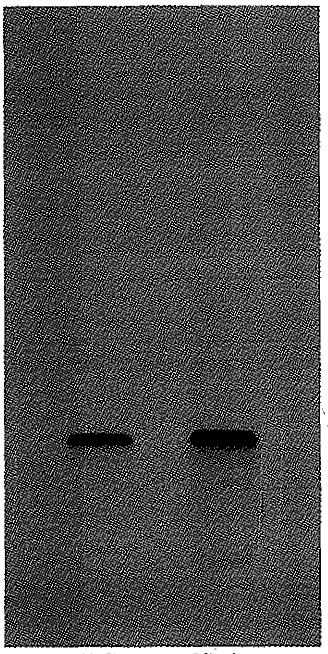
IPBRS TEST
(Under UV light at 366 nm)

IPBRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

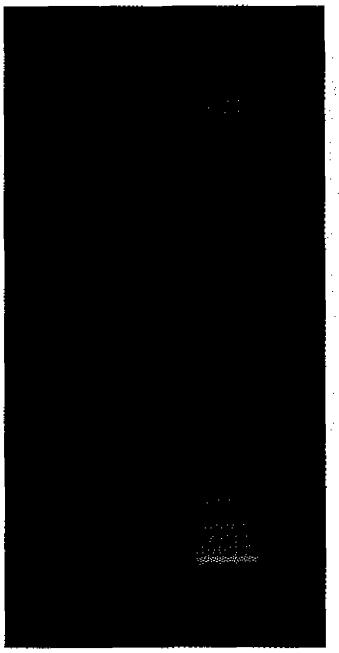
TLC Chromatogram of Yasti

UNAUTHORISED COPY

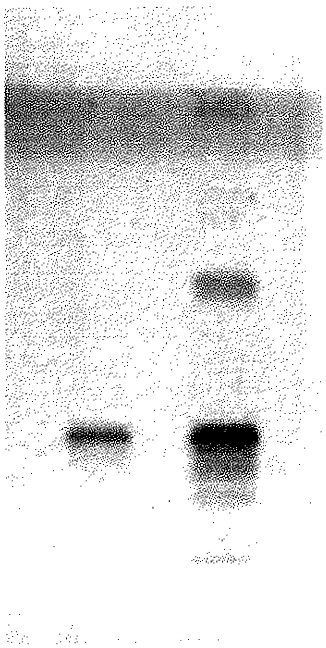
Yasti Dry Extract



IPRS TEST
(Under UV light at 254 nm)



IPRS TEST
(Under UV light at 366 nm)



IPRS TEST
(Under day light after spraying with anisaldehyde sulphuric acid reagent)

TLC Chromatogram of Yasti Dry Extract

UNAUTHORISED COPY

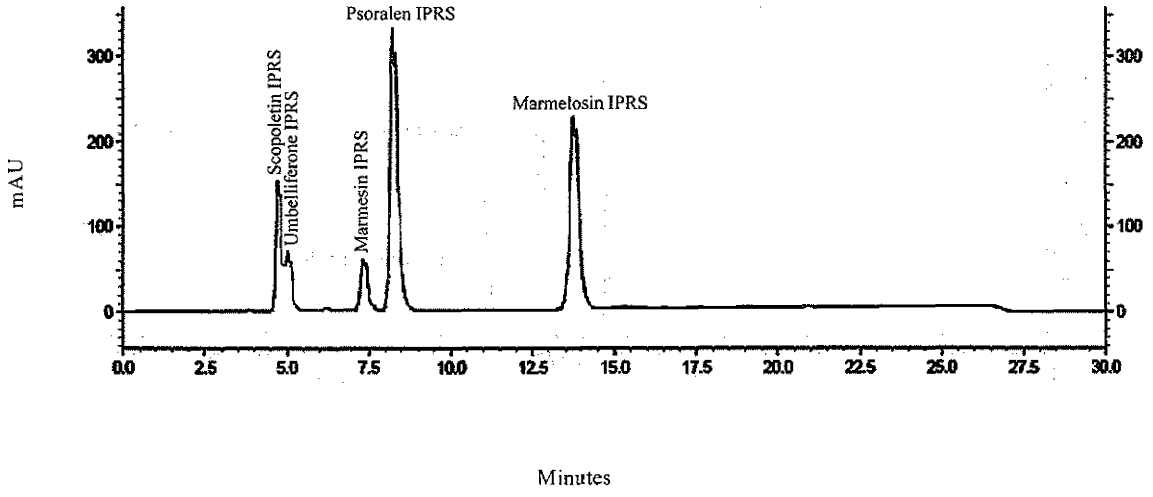
3.3. High Performance Liquid/Gas Chromatograms of Phytopharmaceuticals, Herbs and Herbal Products

In the individual monographs for crude herbs and herbal products test for liquid chromatography or gas chromatography has been given under assay, wherever applicable. Performing this test as per methods prescribed in the individual monographs are to be carried out using a Indian Pharmacopoeia Reference Substance (IPRS) for comparison as specified in the individual monograph. Reference Substance is a compound reported to be present in the plant material, identity and purity of which has been confirmed. Such IPRS may be one of the important chemical compound present in the herbs and herbal products for which a monograph has been specified in the Indian Pharmacopoeia. The IPRS may or may not be an active molecule. Any IPRS issued by the Indian Pharmacopoeia Commission shall be confirmed having not less than 98 per cent w/w purity when tested by relevant measurement techniques. While generally a RS is prepared using isolation and purification techniques from the relevant herbs, it may also be possible to produce a RS by synthesis which may be more economical or easier. Such synthesized compounds shall also be recognized as RS as long as its structural and conformational identity is same as that found in the plant.

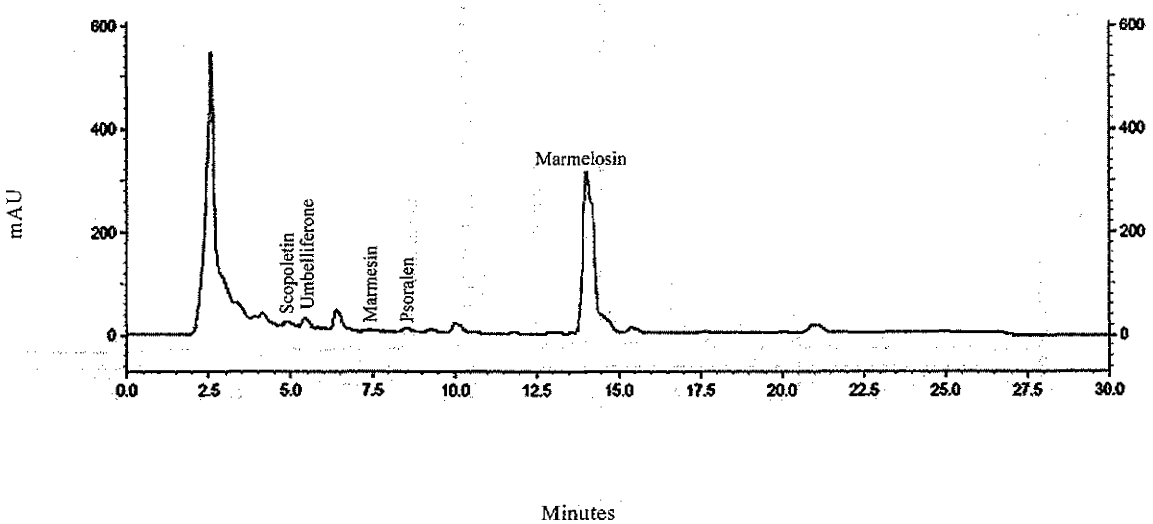
Reproduced below are typical chromatograms of 3.3.1. Phytopharmaceuticals, 3.3.2. Herbs and herbal products for which a monograph appears in this edition of IP. Each typical chromatogram bears the name of the monograph at left hand top position, while the name of IPRS and the test material below it. Such reproduction consists of two chromatograms for each monograph. The first one would be the reproduction when a IPRS has been run as per the method prescribed in the monograph. The second part would consist of the chromatogram obtained when a substance under examination has been run as per the method prescribed in the monographs. In both of them the RT at which the peak of the IPRS would be marked. These have been provided by individual participating organisations which assisted in development of monographs and methods have undergone collaborative testing.

These chromatograms have been provided for guidance purposes only, comparison to same may be adopted routinely, though not for regulatory purposes as they do not form the part of the monograph.

Aegle marmelos PPI

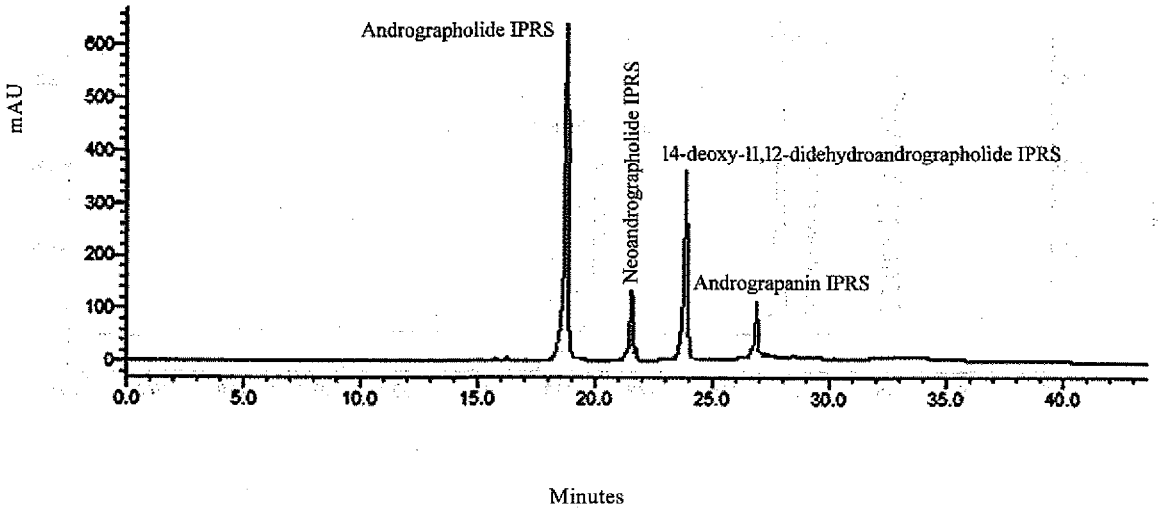


HPLC Chromatogram of scopoletin IPRS, umbelliferone IPRS, marmesin IPRS, psoralen IPRS, marmelosin IPRS

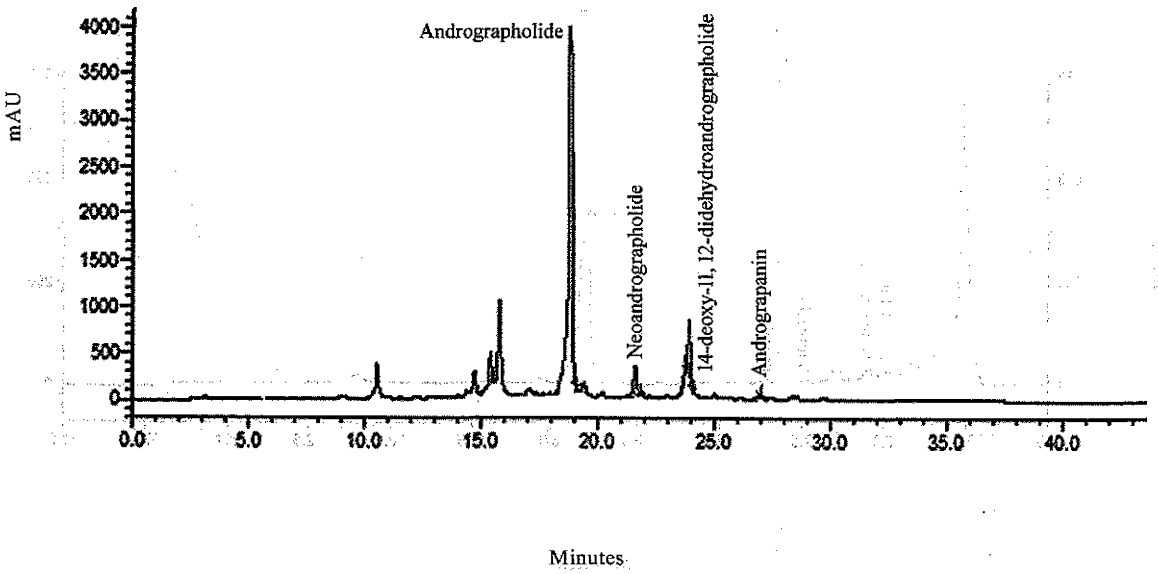


HPLC Chromatogram of Aegle marmelos PPI

Andrographis paniculata PPI

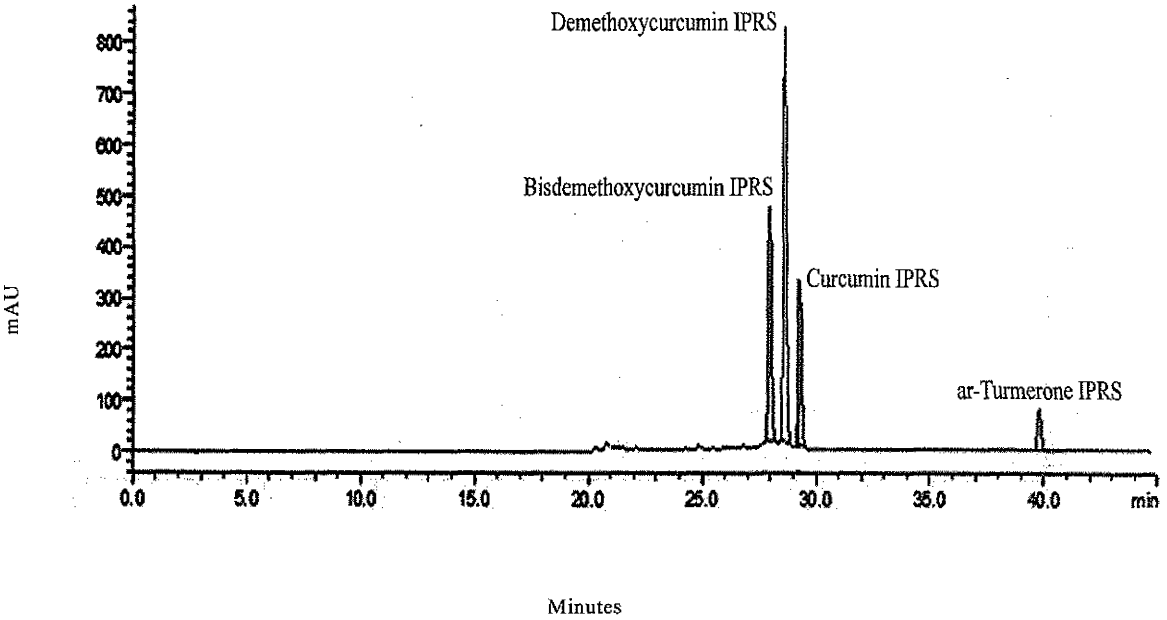


HPLC Chromatogram of andrographolide IPRS, neoandrographolide IPRS, 14-deoxy-11,12-didehydroandrographolide IPRS and andrograpanin IPRS



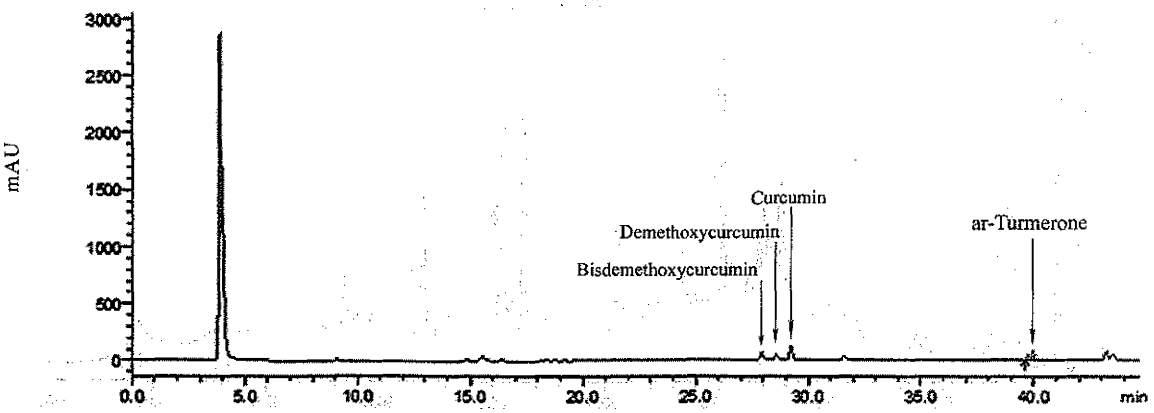
HPLC Chromatogram of Andrographis paniculata PPI

Curcuma longa PPI



Minutes

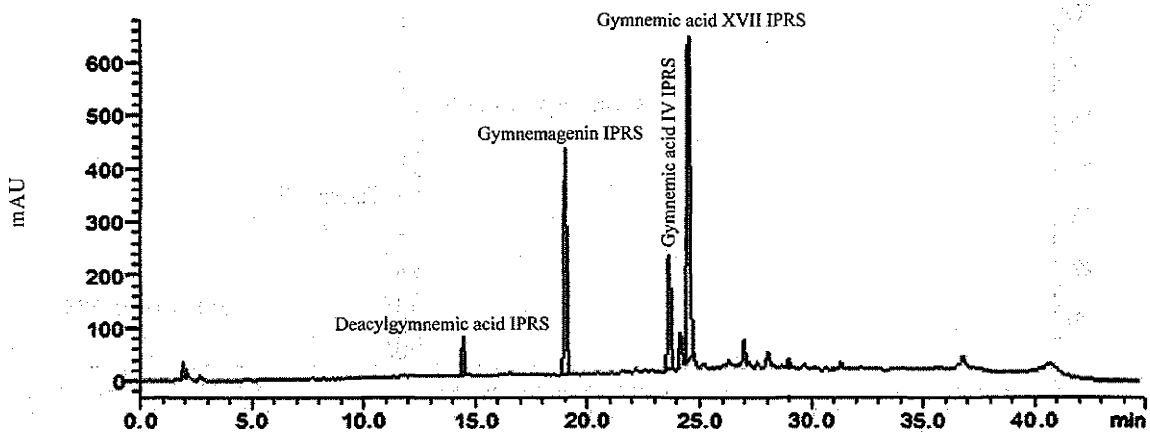
HPLC Chromatogram of bisdemethoxycurcumin IPRS, demethoxycurcumin IPRS, curcumin IPRS and ar-turmerone IPRS



Minutes

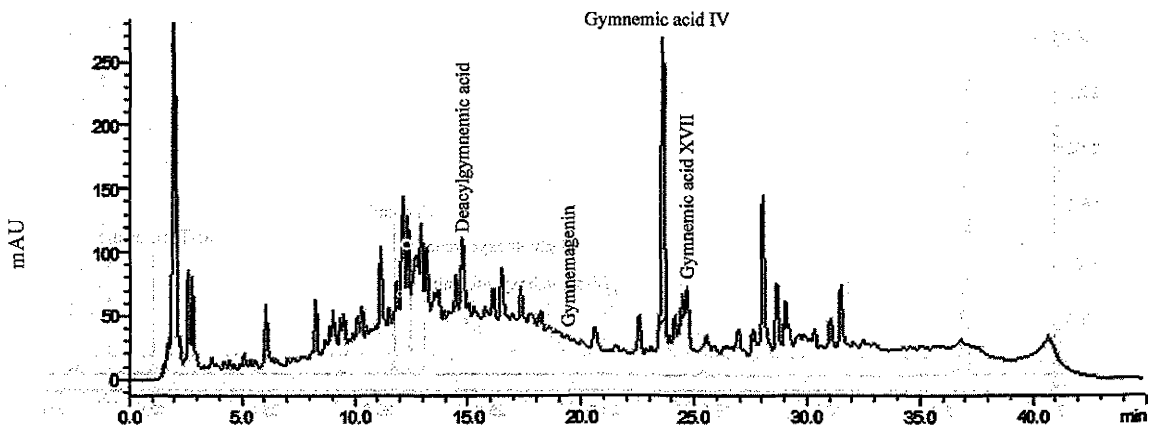
HPLC Chromatogram of Curcuma longa PPI

Gymnema sylvestre PPI



Minutes

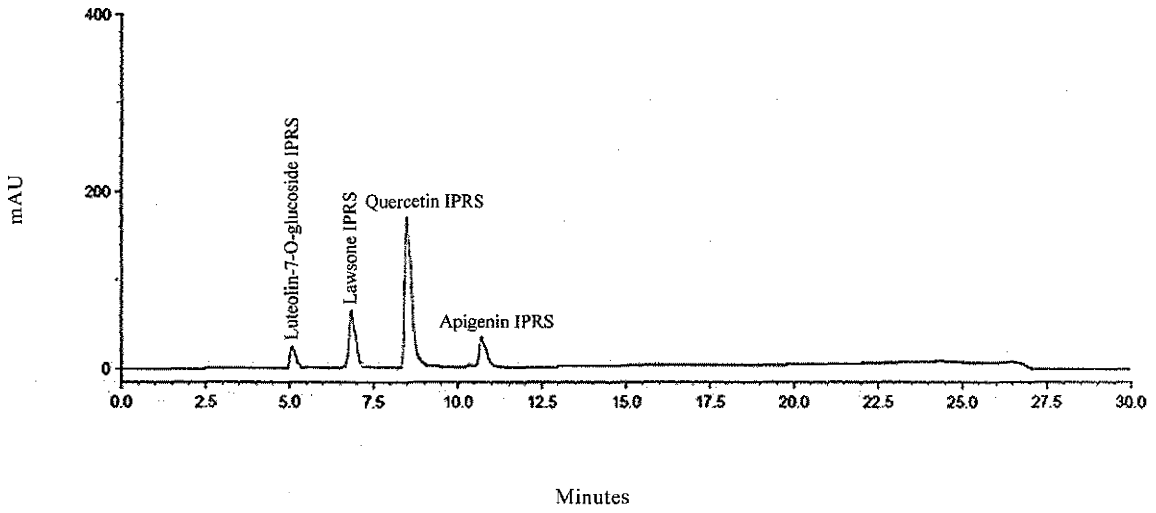
HPLC Chromatogram of deacylgymnemic acid IPRS, gymnemagenin IPRS, gymnemic acid IV IPRS, gymnemic acid XVII IPRS



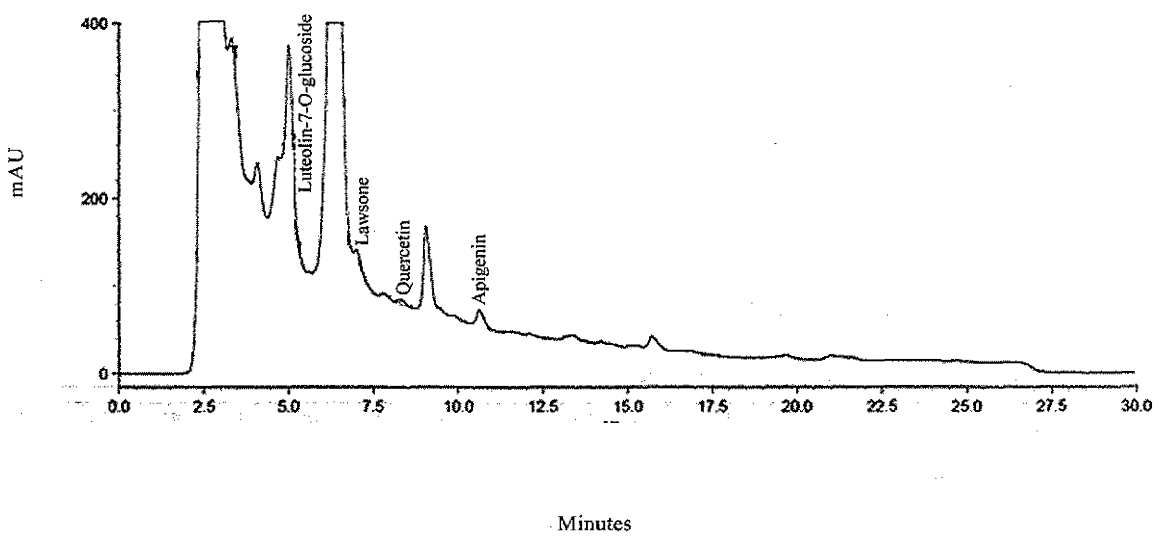
Minutes

HPLC Chromatogram of Gymnema sylvestre PPI

Lawsonia inermis PPI

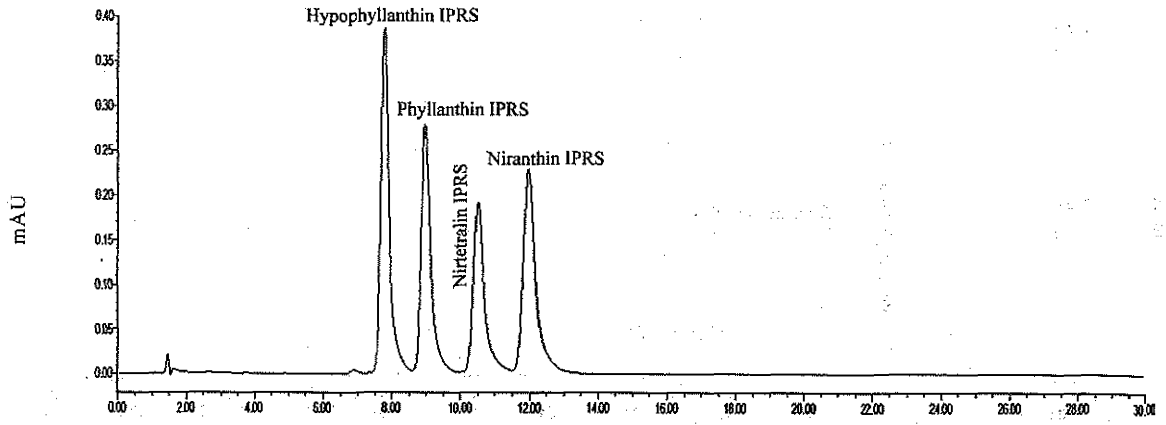


HPLC Chromatogram of luteolin-7-O-glucoside IPRS, lawsone IPRS, quercetin IPRS and apigenin IPRS

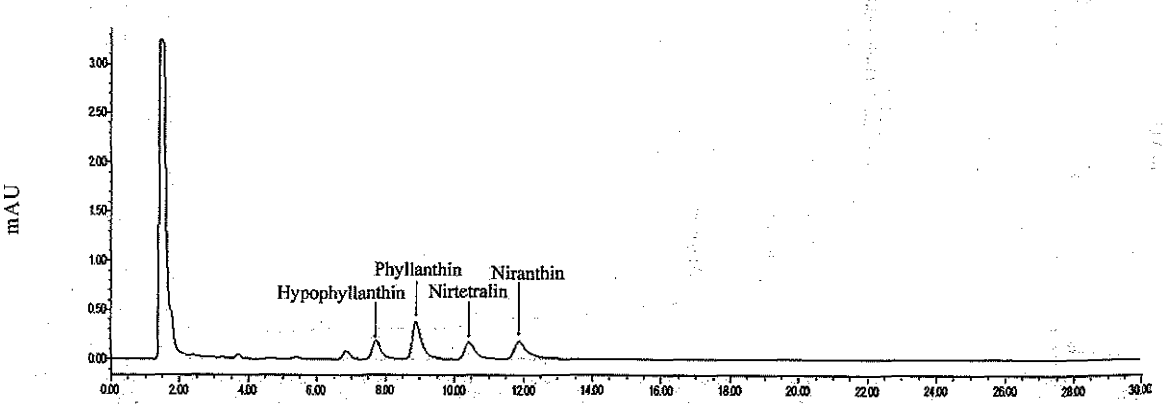


HPLC Chromatogram of Lawsonia inermis PPI

Phyllanthus amarus PPI

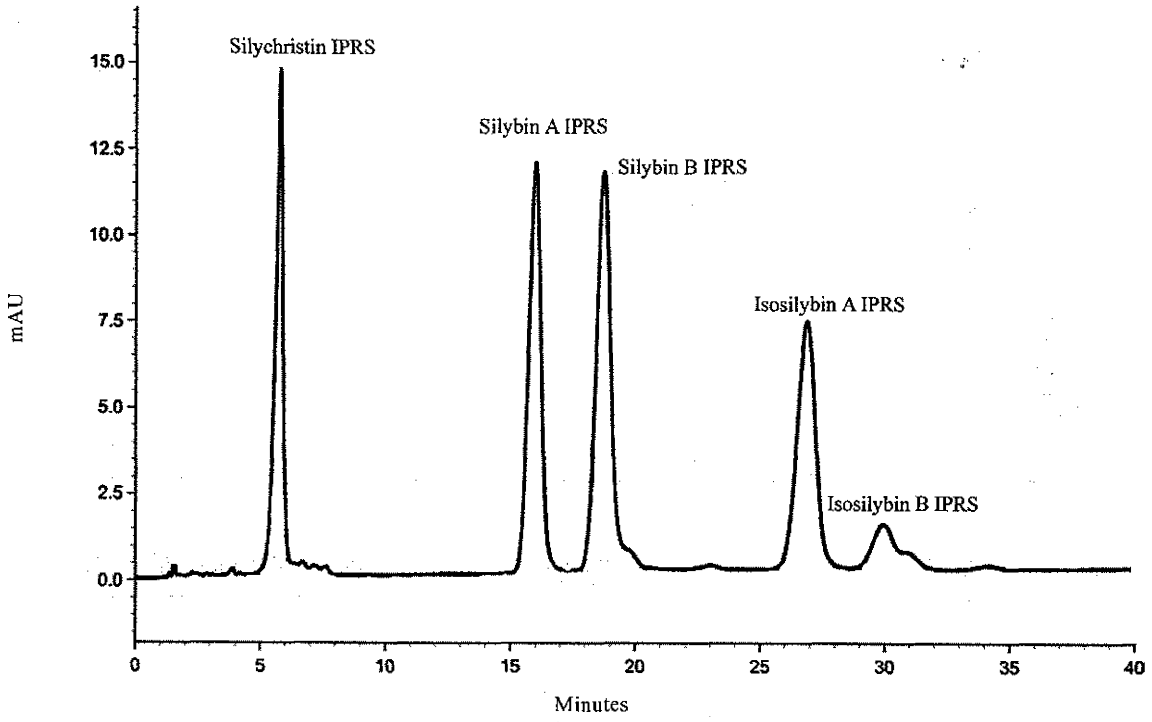


HPLC Chromatogram of hypophyllanthin IPRS, phyllanthin IPRS, nirtetralin IPRS, niranthin IPRS

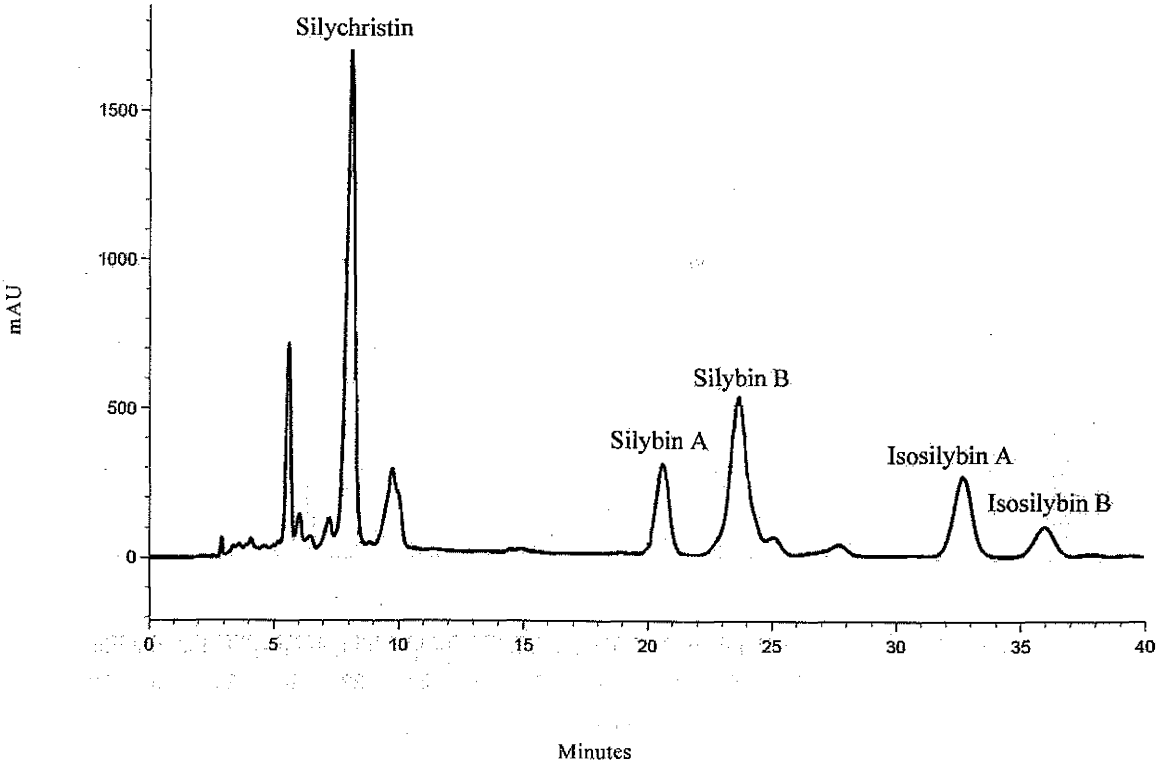


HPLC Chromatogram of Phyllanthus amarus PPI

Silybum marianum Dry Extract PPI

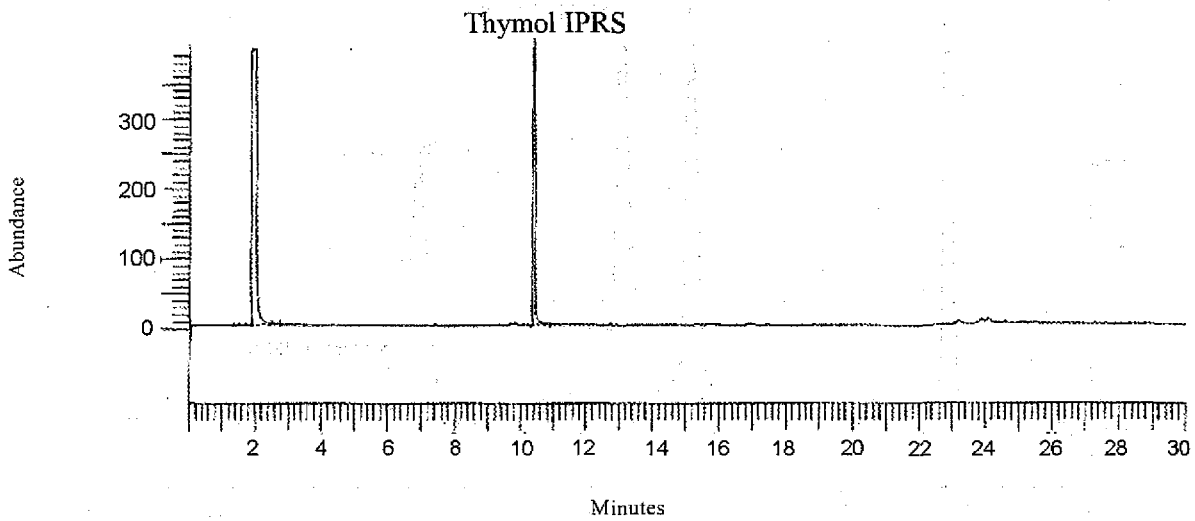


HPLC Chromatogram of silychristin IPRS, silybin A IPRS, silybin B IPRS, isosilybin A IPRS and isosilybin B IPRS

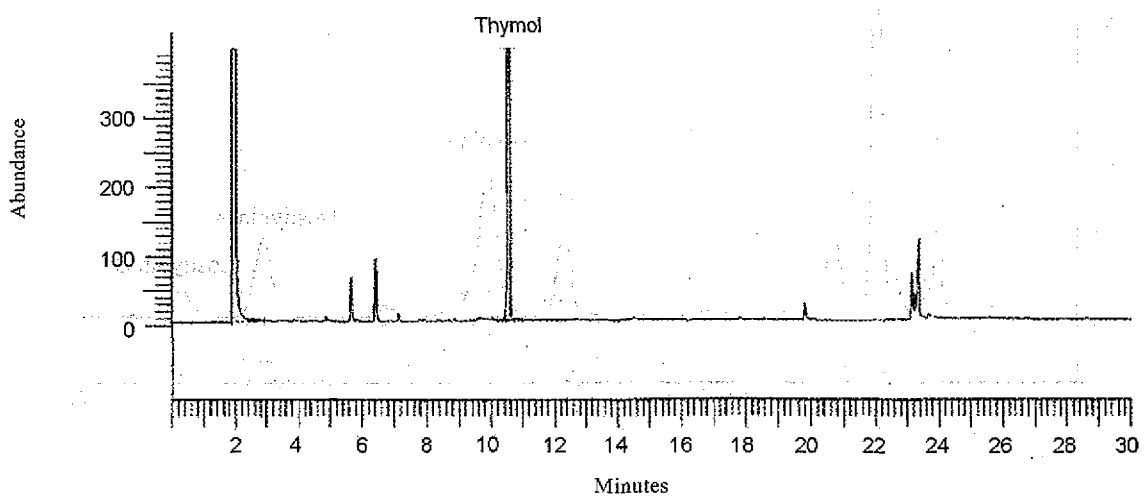


HPLC Chromatogram of Silybum marianum Dry Extract

Ajwain

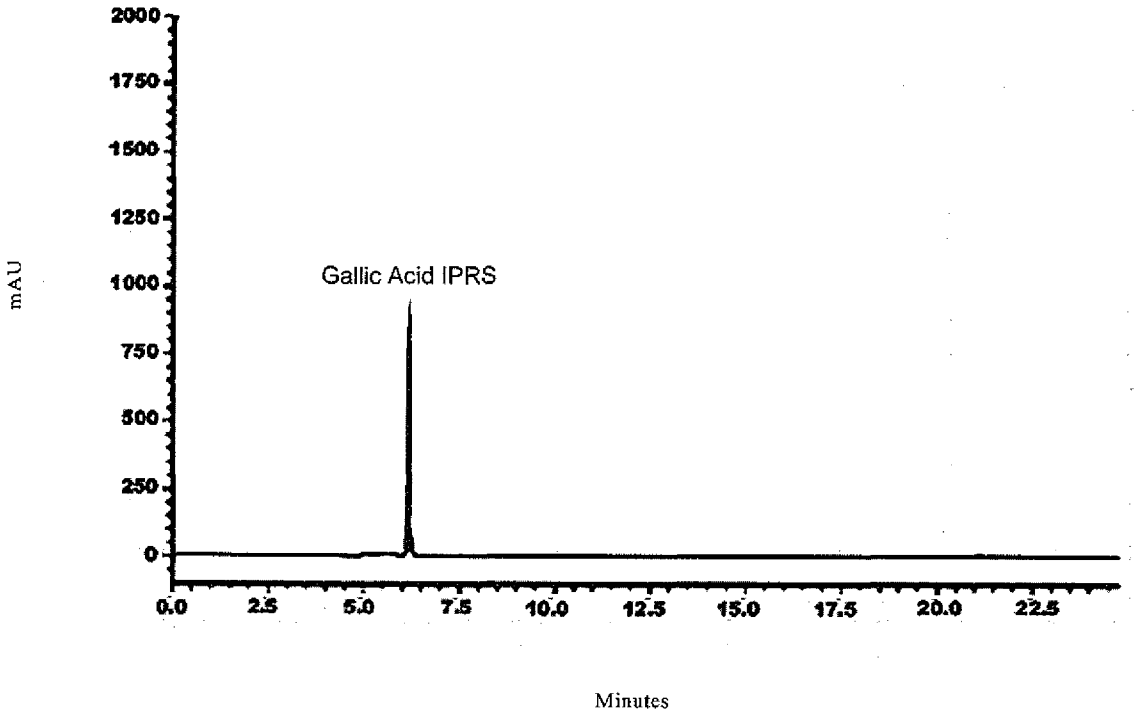


Gas Chromatogram of thymol IPRS

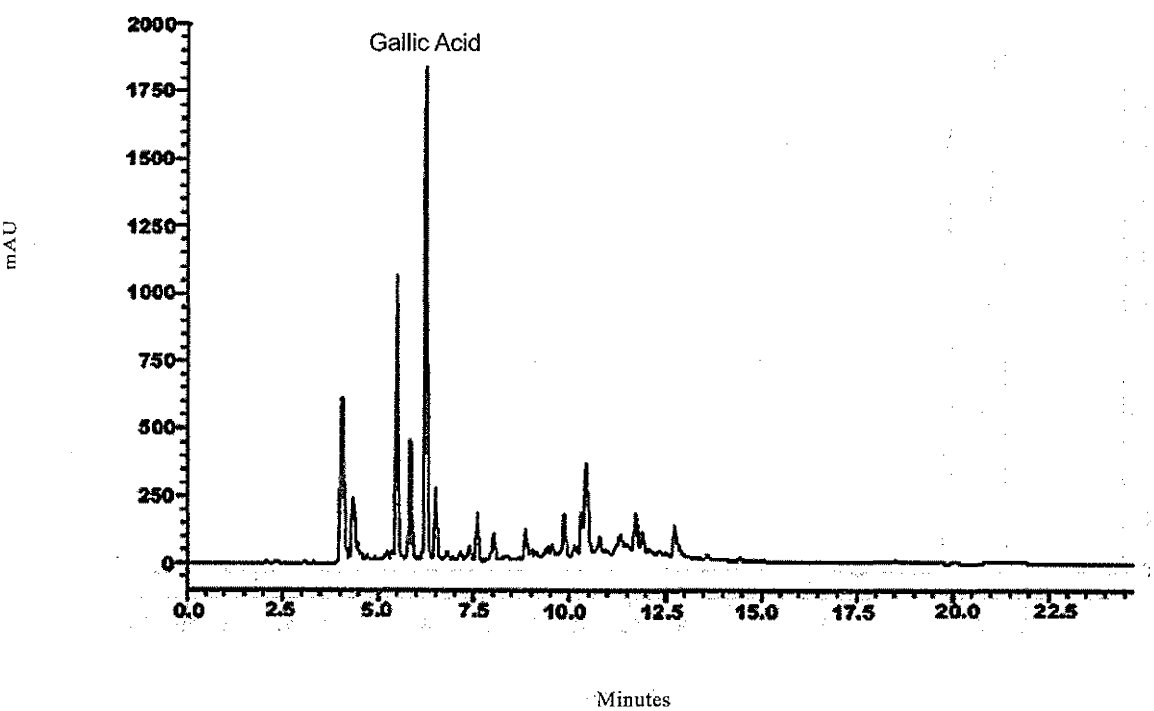


Gas Chromatogram of Ajwain

Amalaki

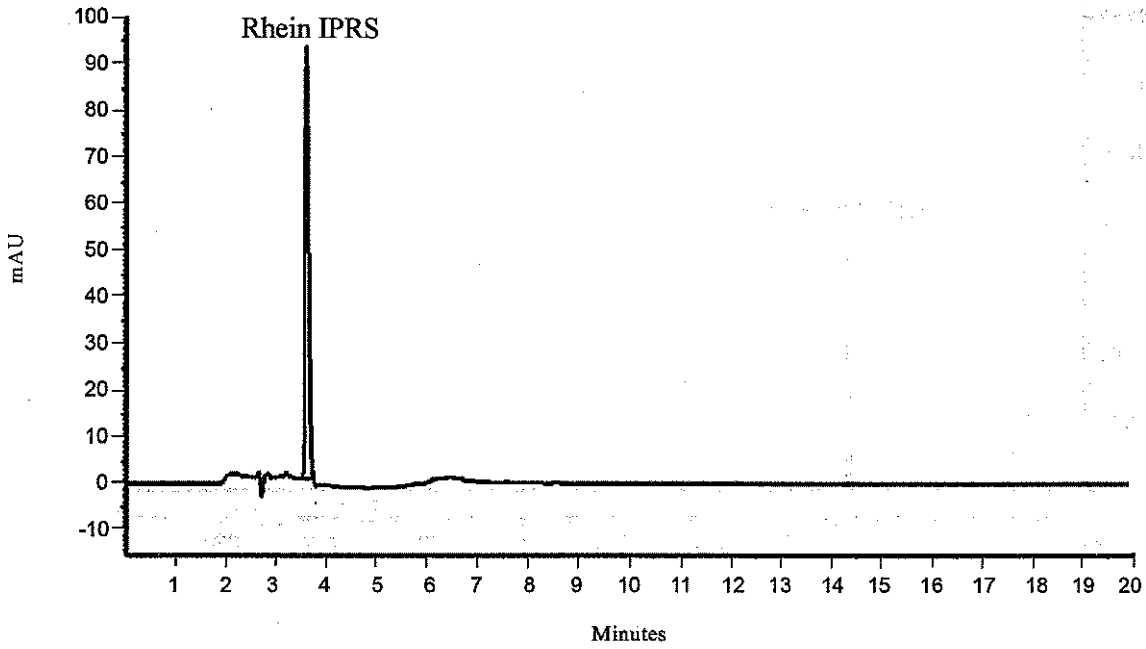


HPLC Chromatogram of gallic acid IPRS

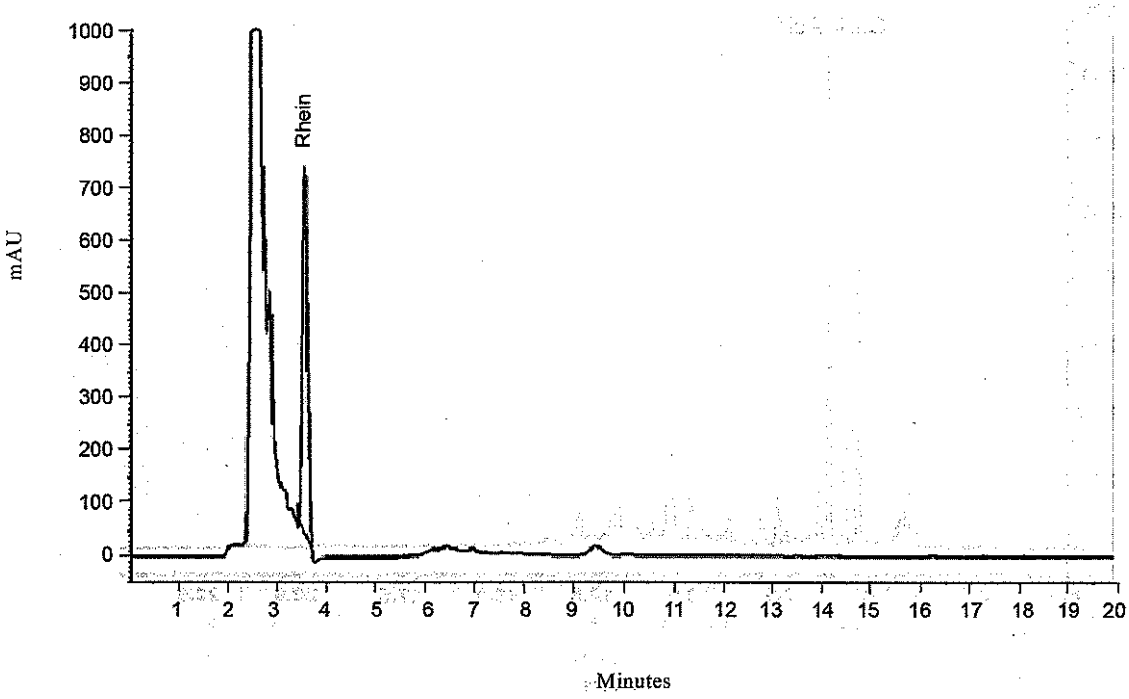


HPLC Chromatogram of Amalaki

Amaltas

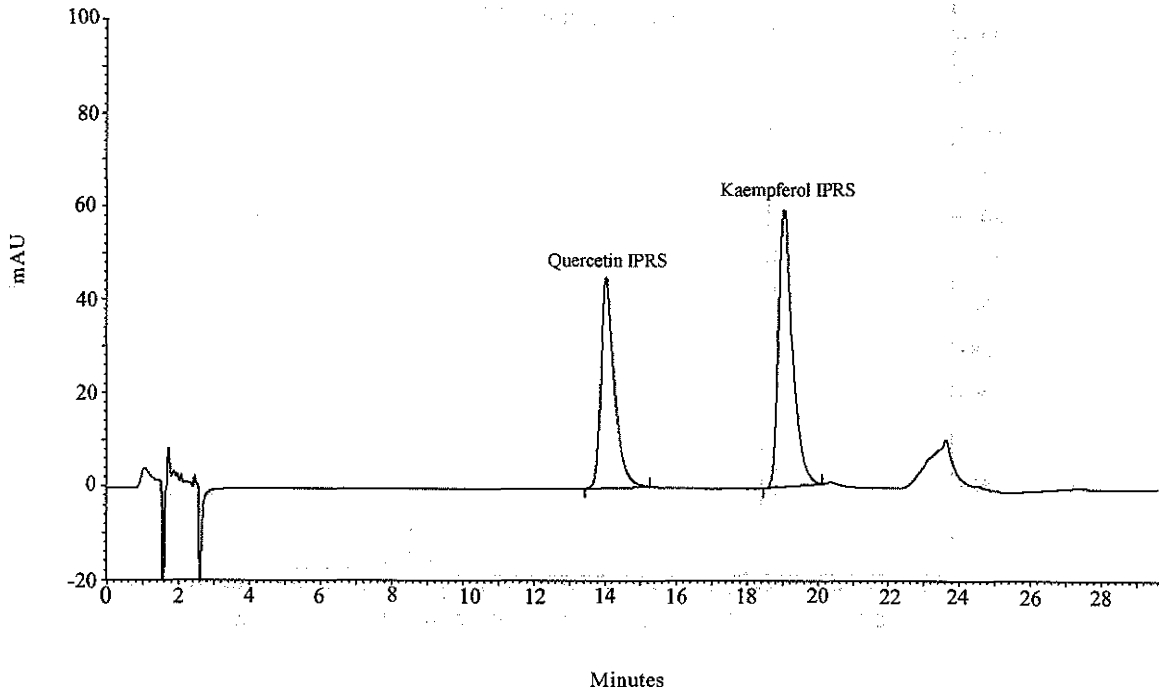


HPLC Chromatogram of rhein IPRS

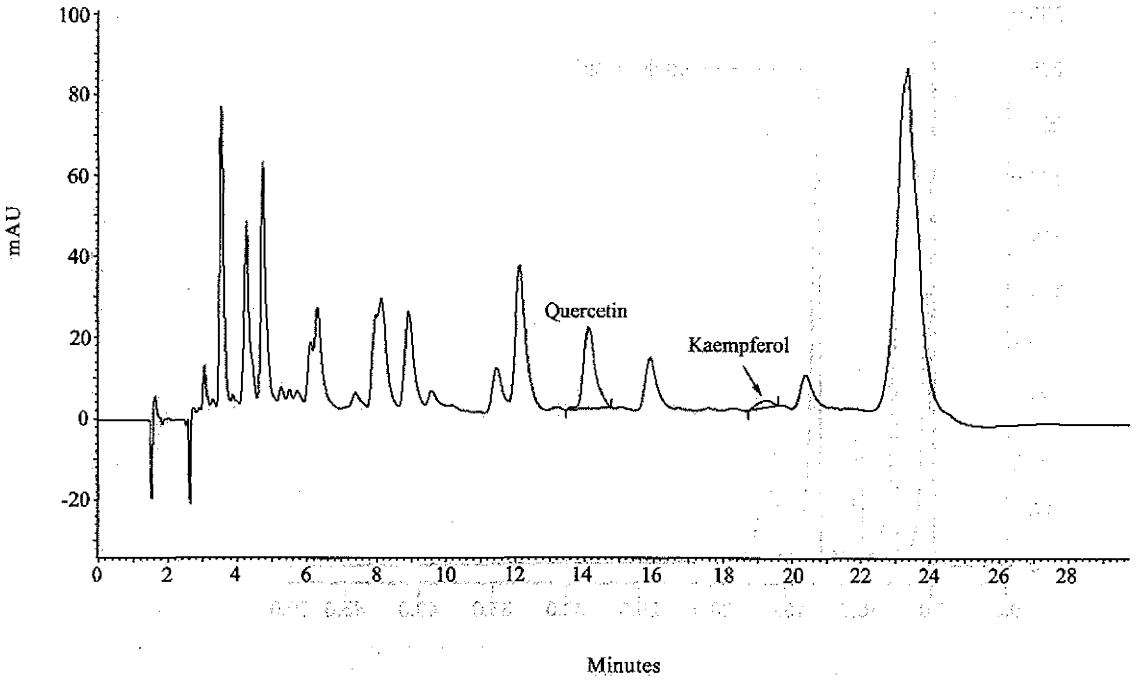


HPLC Chromatogram of Amaltas

Amarbel

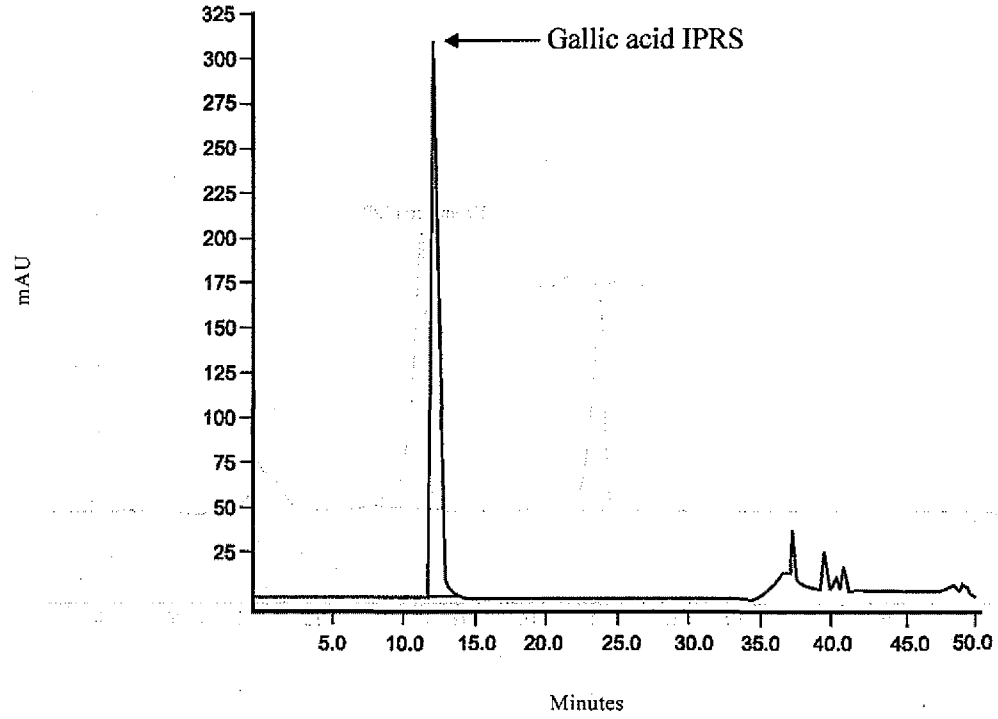


HPLC Chromatogram of quercetin IPRS and kaempferol IPRS

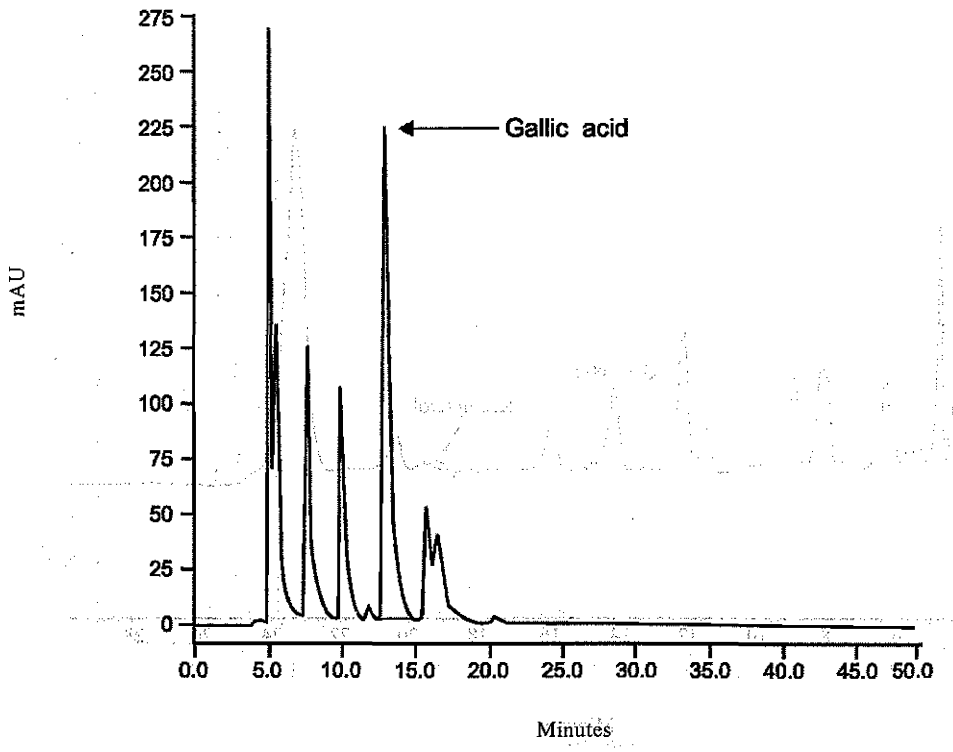


HPLC Chromatogram of Amarbel

Amla Juice Powder

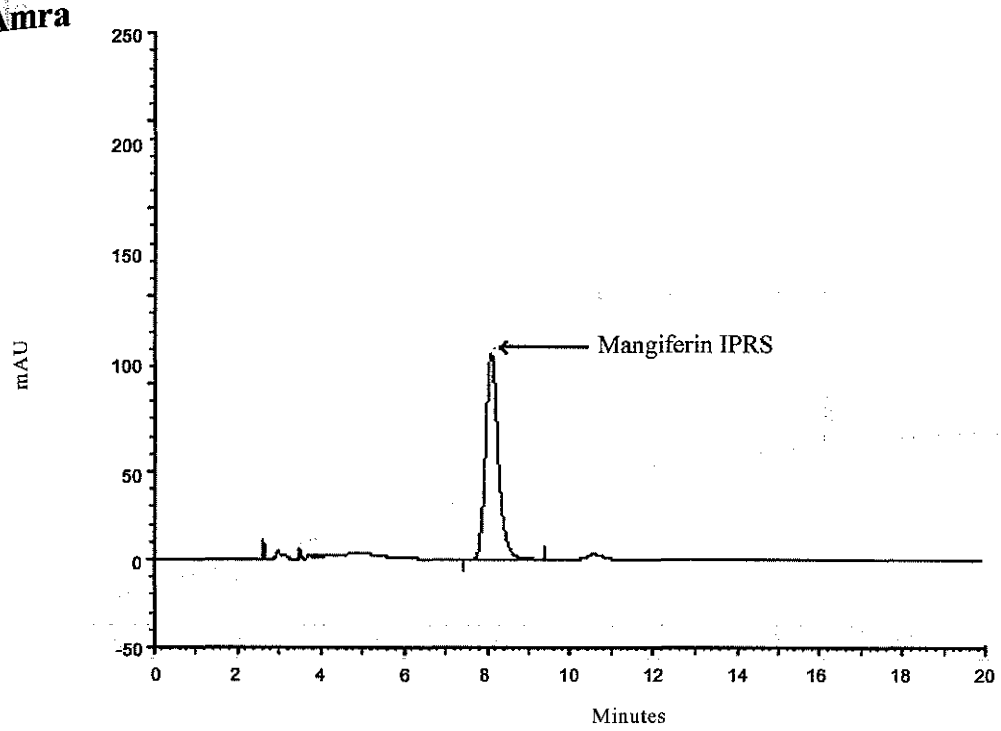


HPLC Chromatogram of gallic acid IPRS

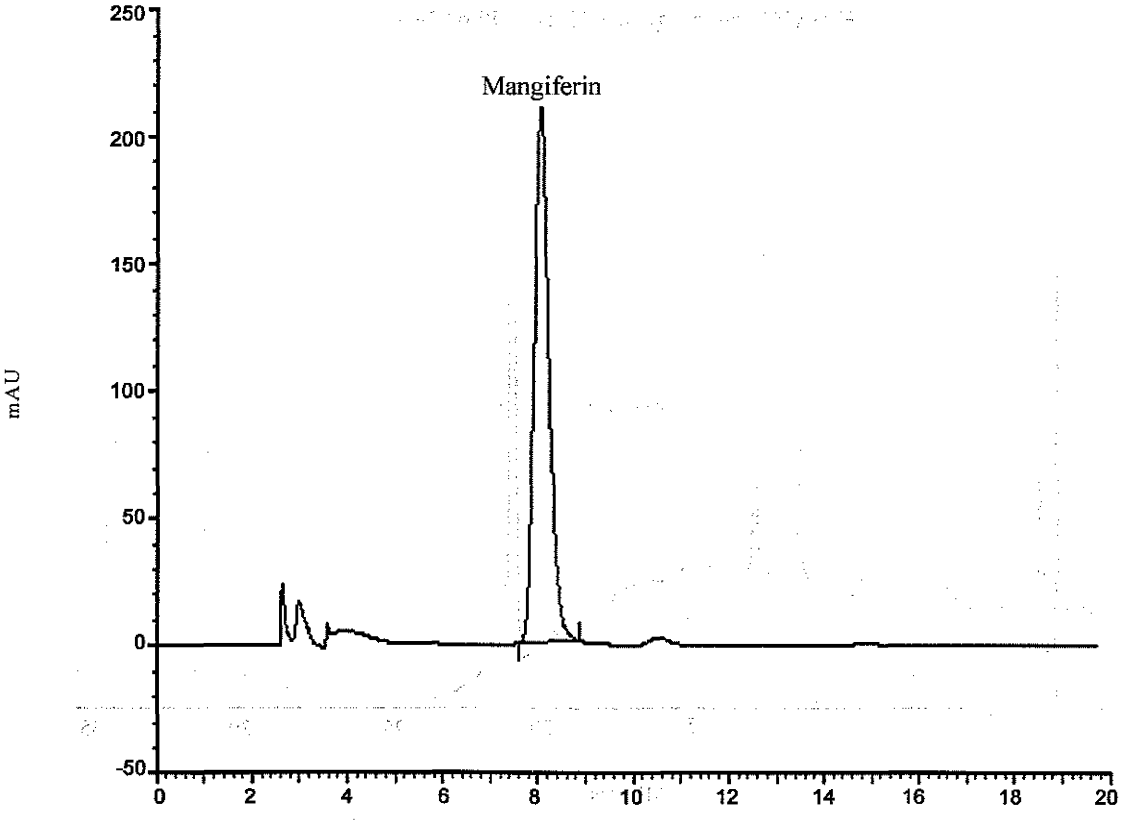


HPLC Chromatogram of Amla Juice Powder

Amra

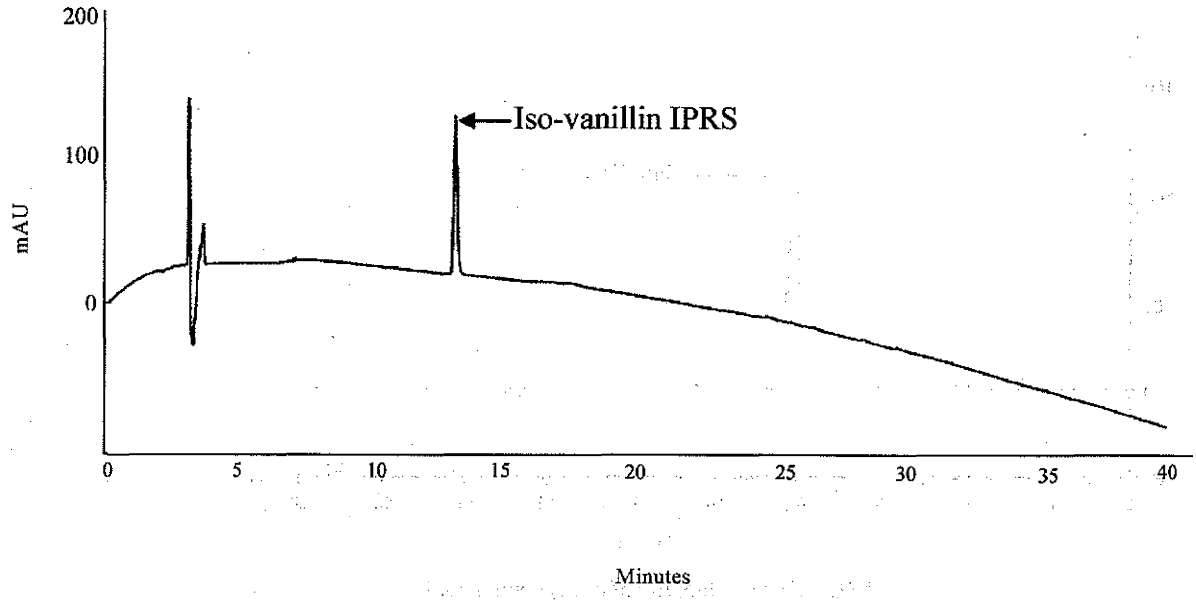


HPLC Chromatogram of mangiferin IPRS

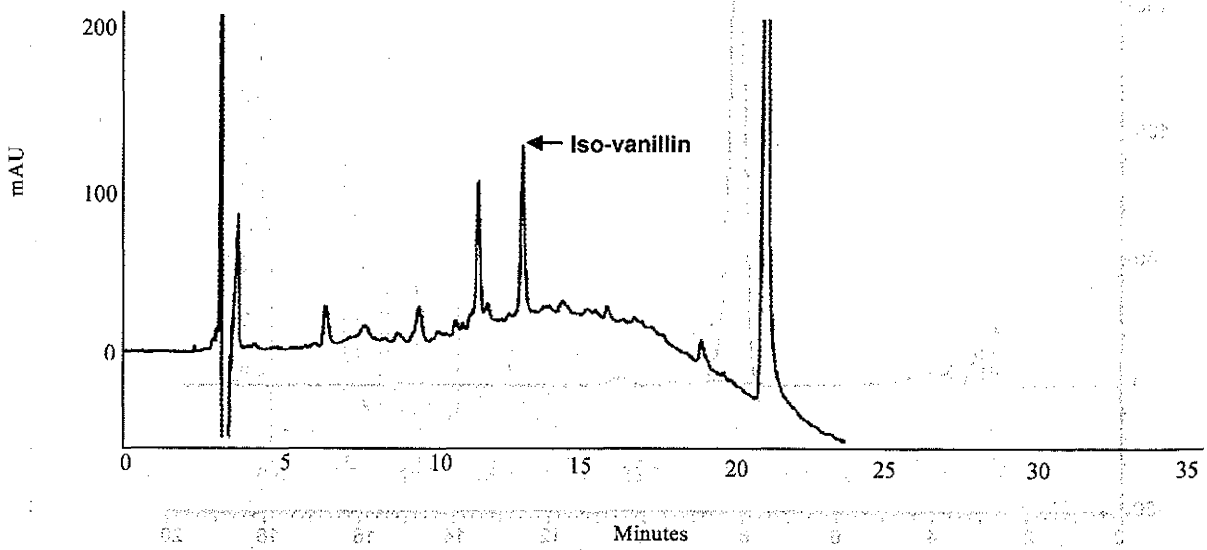


HPLC Chromatogram of Amra

Anantmula

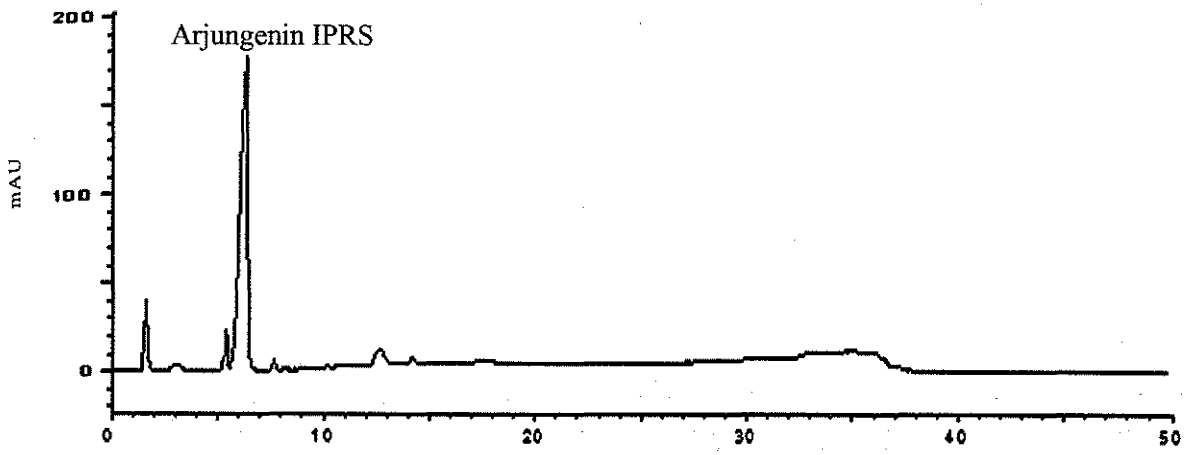


HPLC Chromatogram of iso-vanillin IPRS

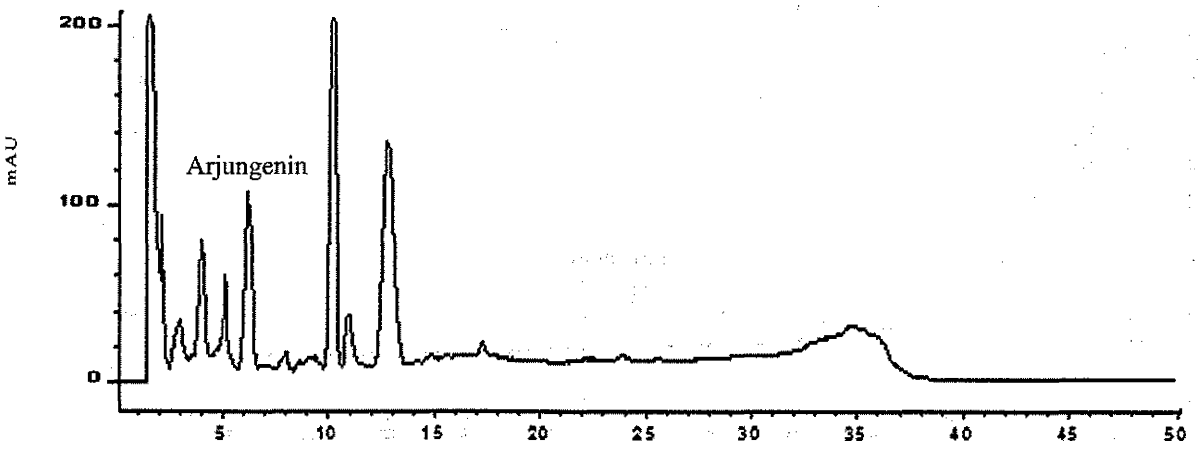


HPLC Chromatogram of Anantmula

Arjuna

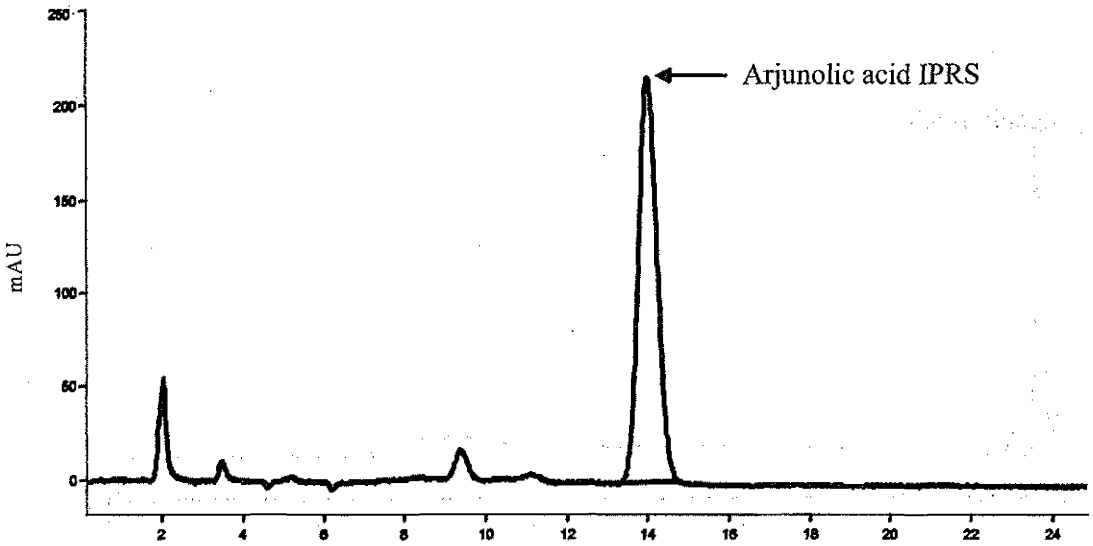


HPLC Chromatogram of arjungenin IPRS

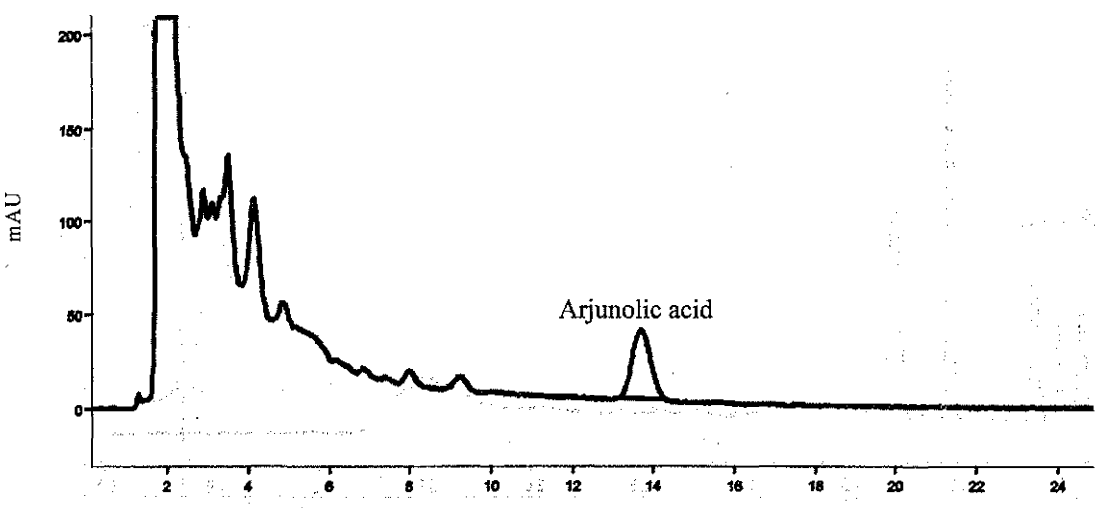


HPLC Chromatogram of Arjuna

Arjuna Dry Extract

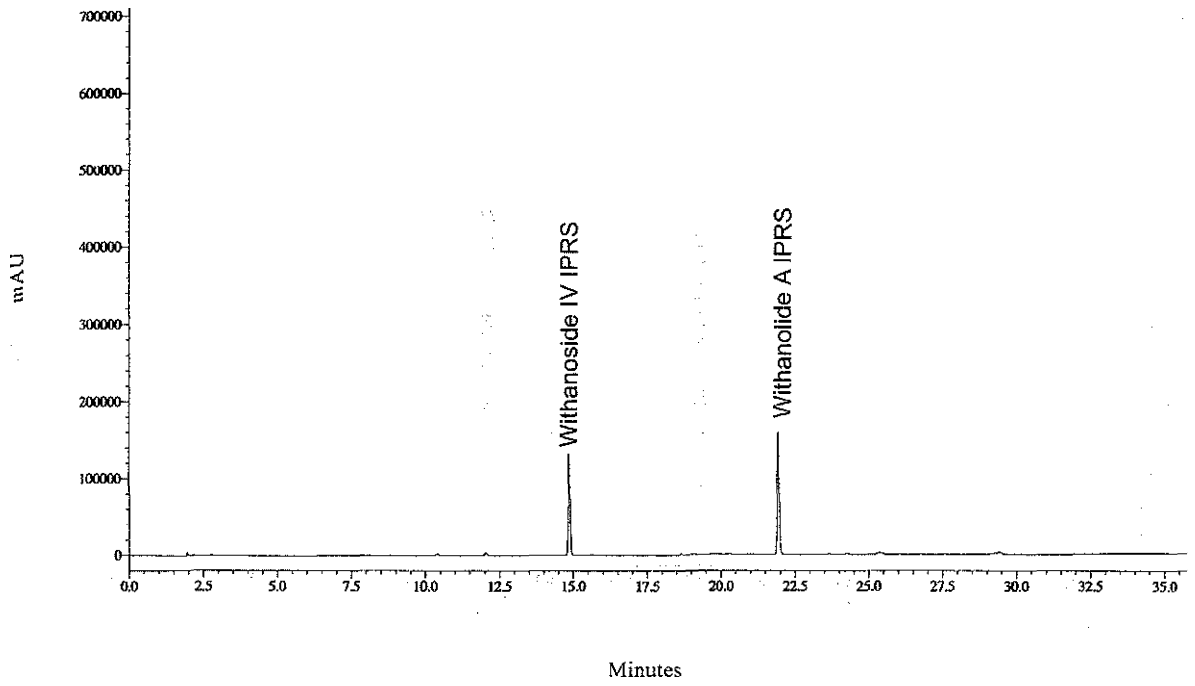


HPLC Chromatogram of arjunolic acid IPRS

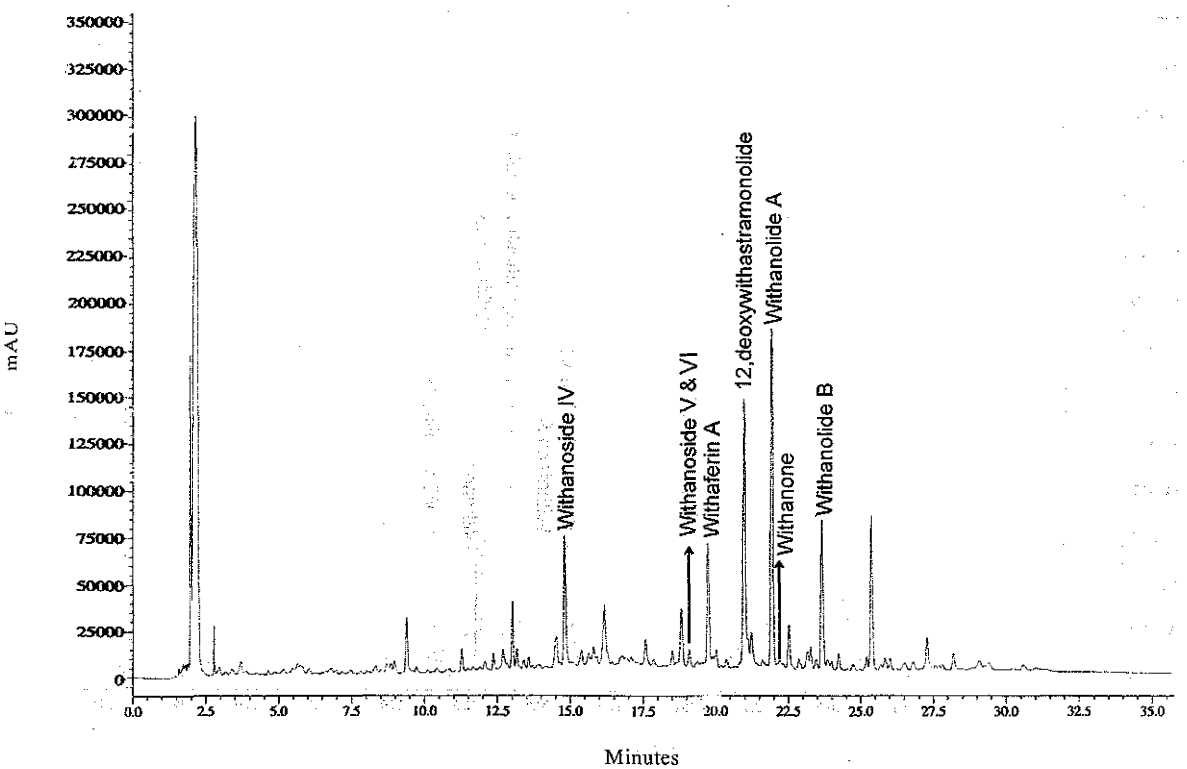


HPLC Chromatogram of Arjuna Dry Extract

Ashwagandha

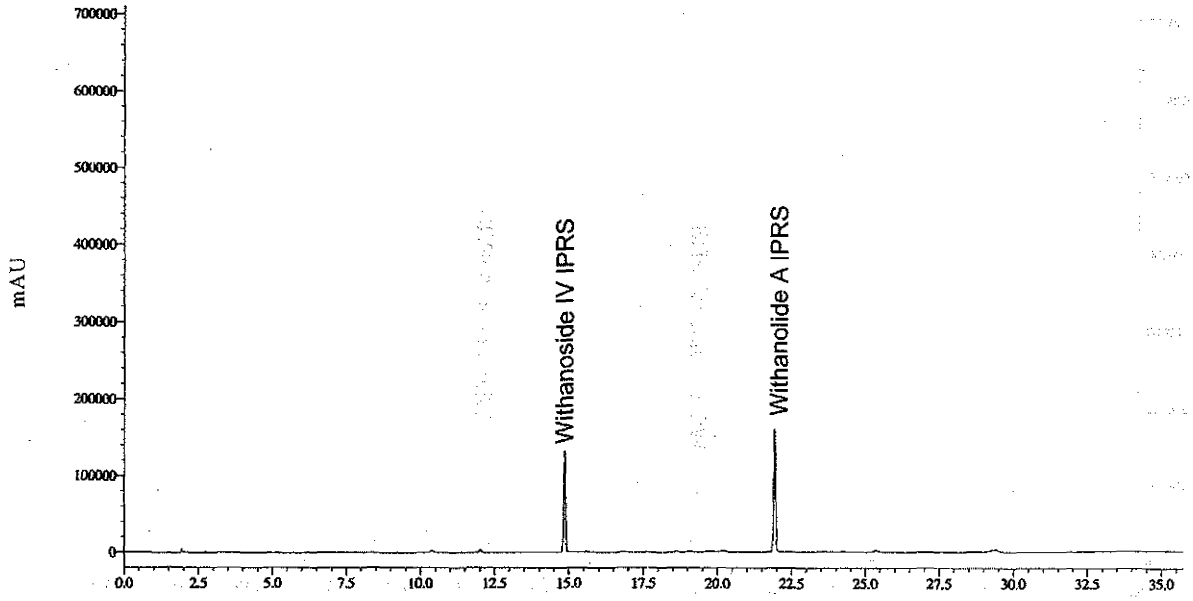


HPLC Chromatogram of withanoside IV IPRS and withanolide A IPRS

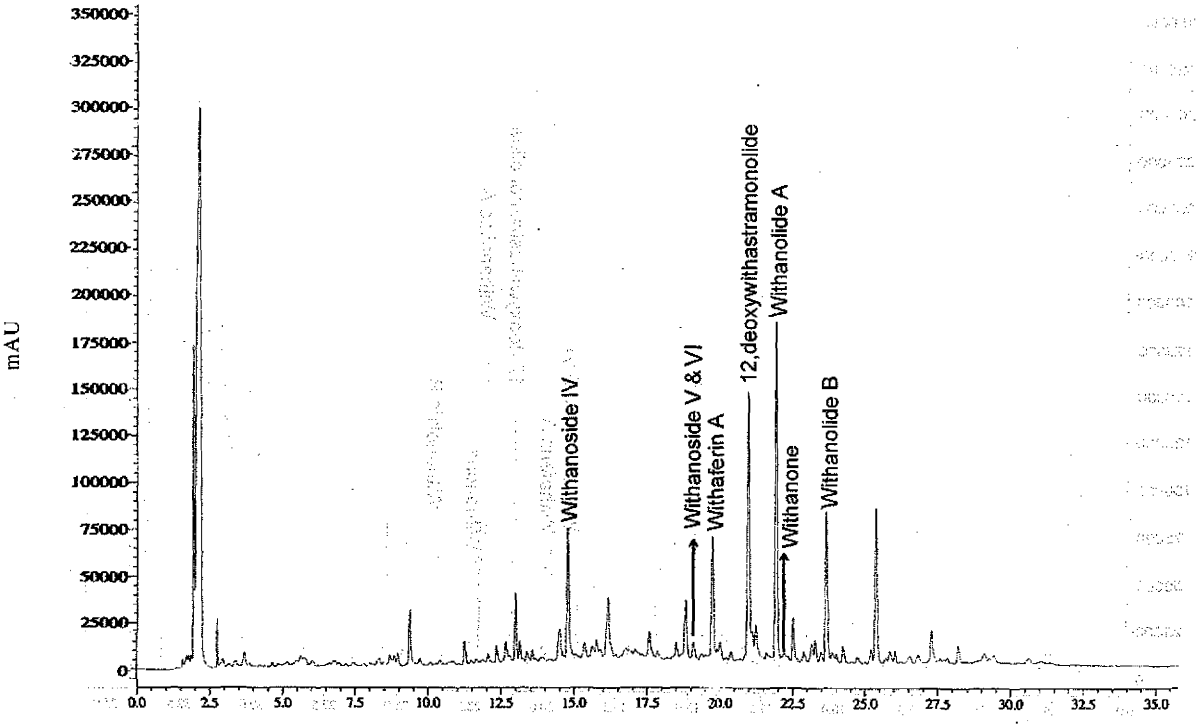


HPLC Chromatogram of Ashwagandha

Ashwagandha Dry Extract

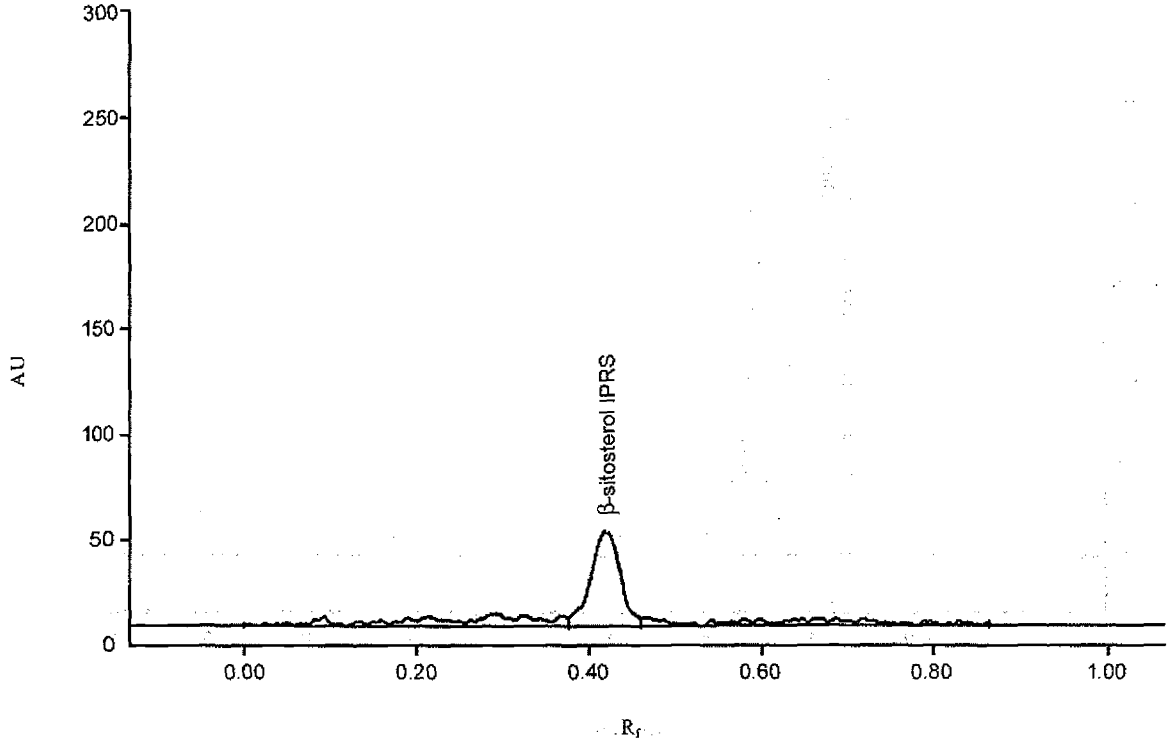


HPLC Chromatogram of withanoside IV IPRS and withanolide A IPRS

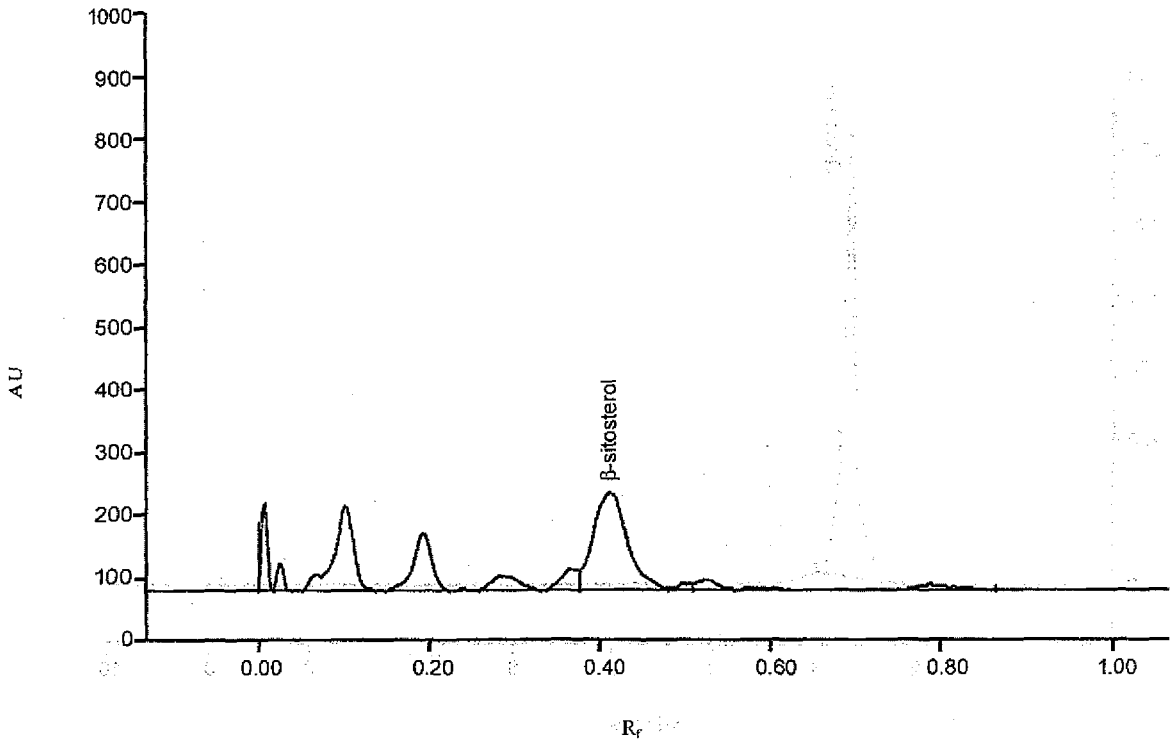


HPLC Chromatogram of Ashwagandha Dry Extract

Asoka

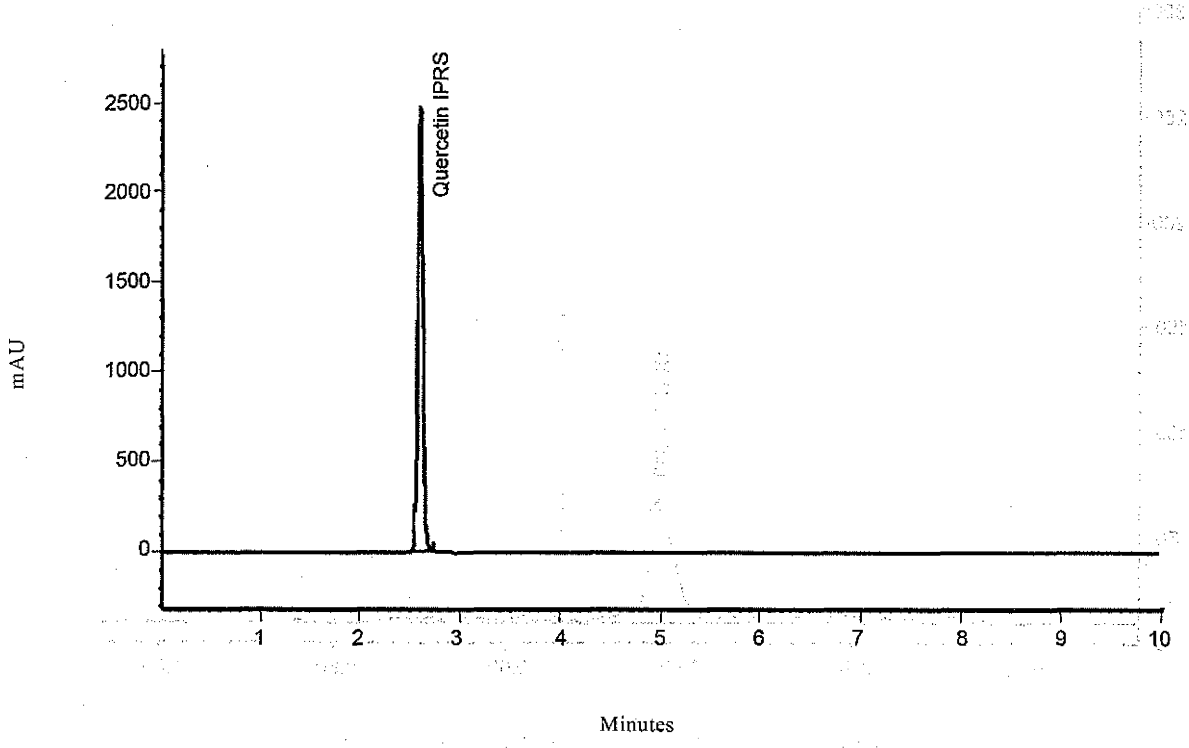


HPTLC Chromatogram of β-sitosterol

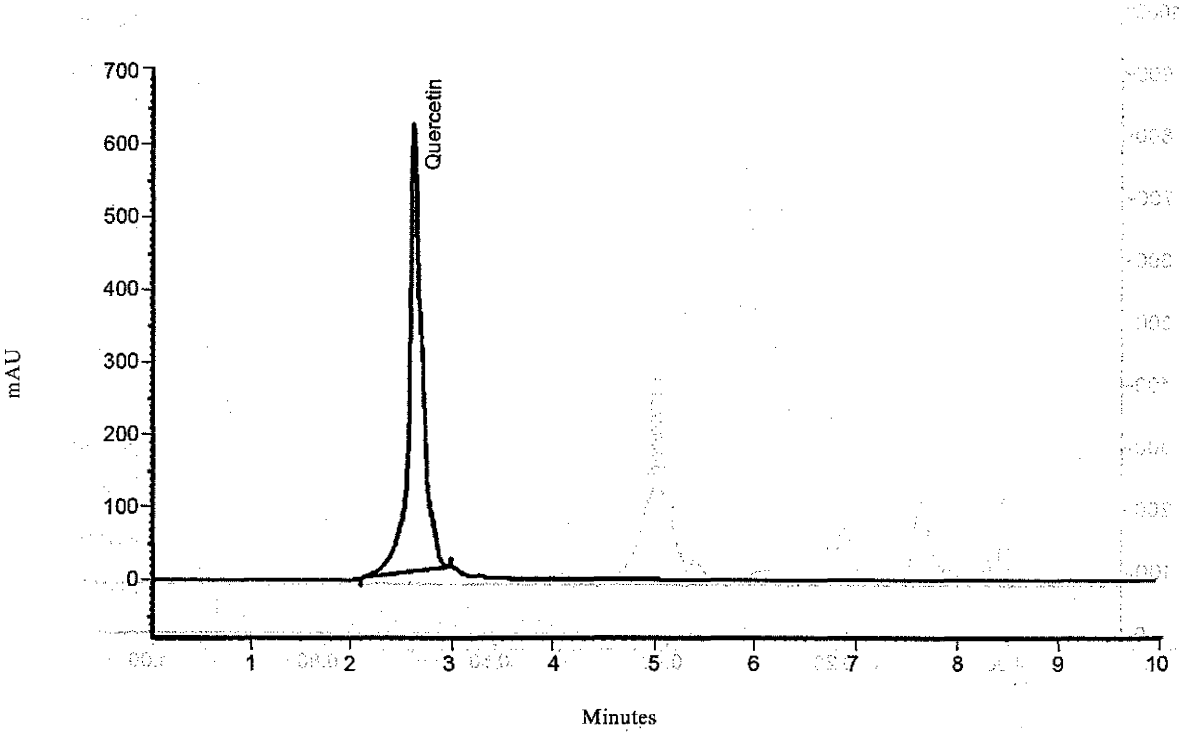


HPTLC Chromatogram of Asoka

Asthisamhrta

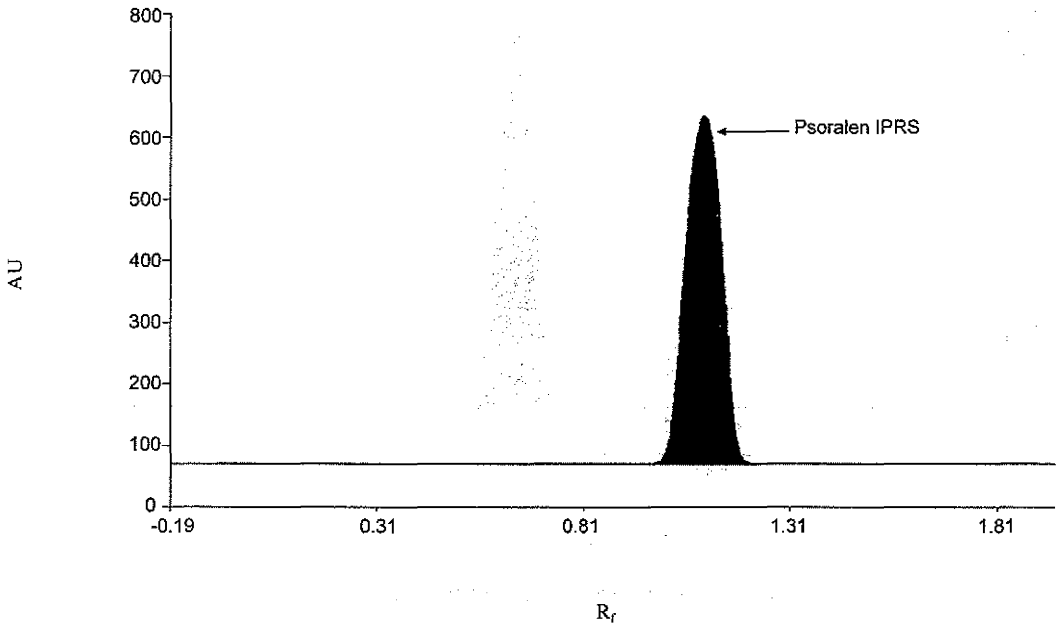


HPLC Chromatogram of quercetin IPRS

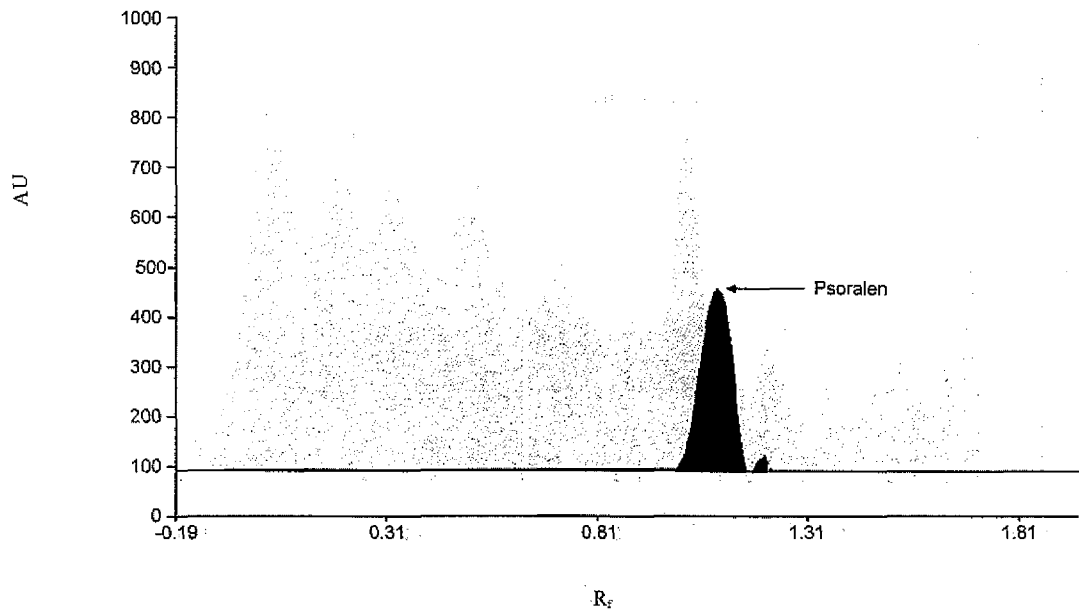


HPLC Chromatogram of Asthisamhrta

Bakuci

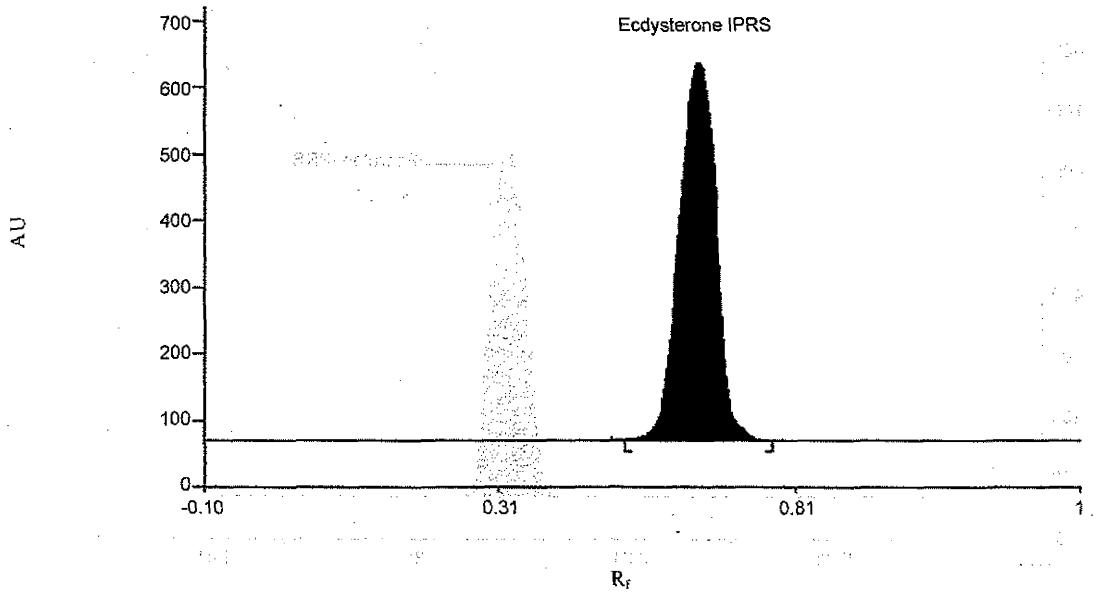


HPTLC Chromatogram of psoralen IPRS

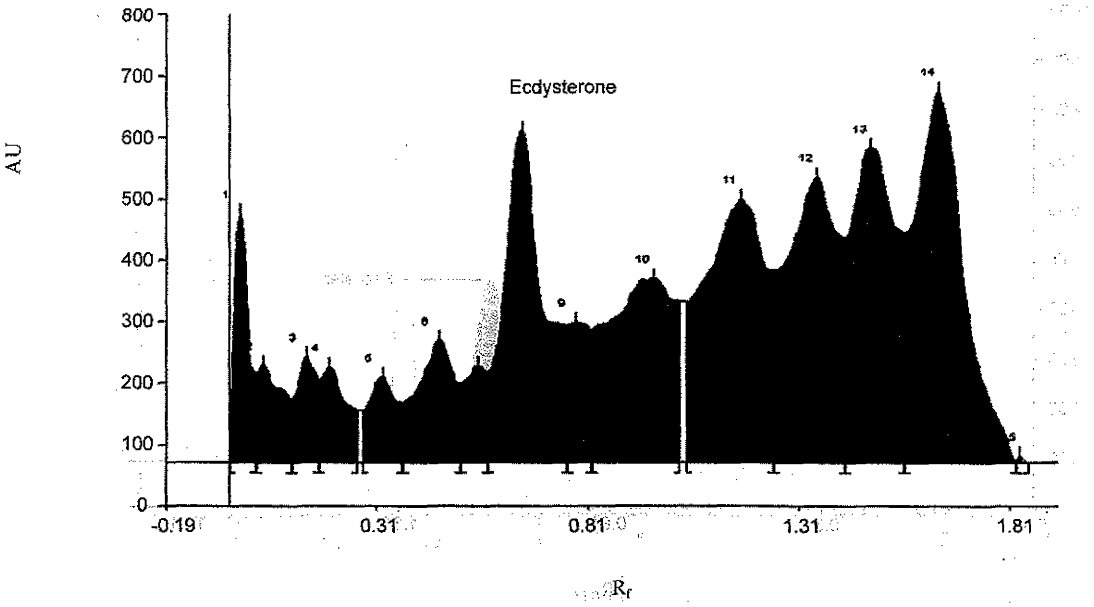


HPTLC Chromatogram of Bakuci

Bala

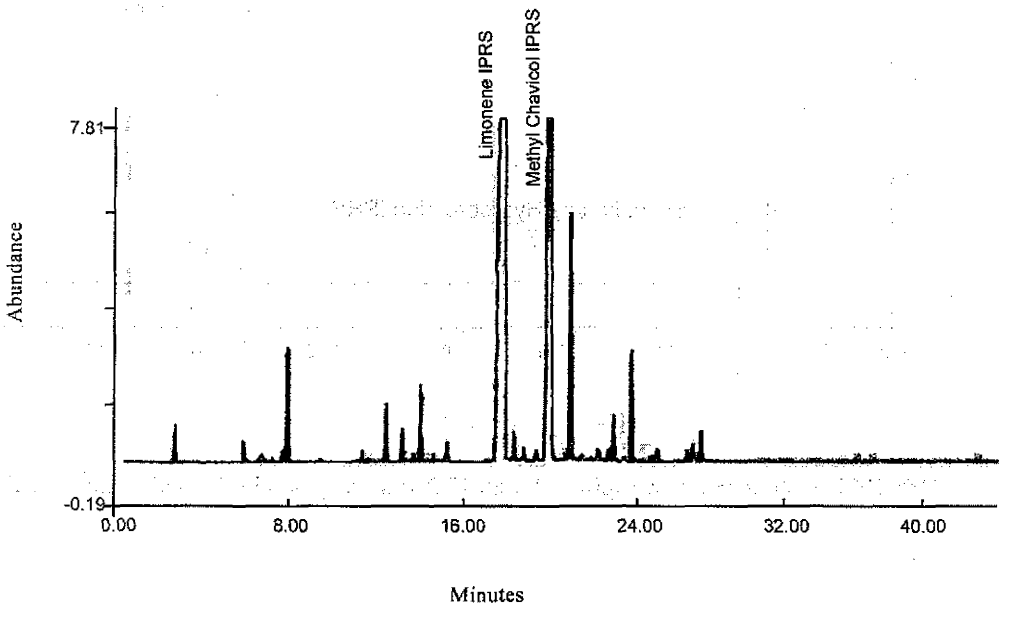


HPTLC Chromatogram of ecdysterone IPRS



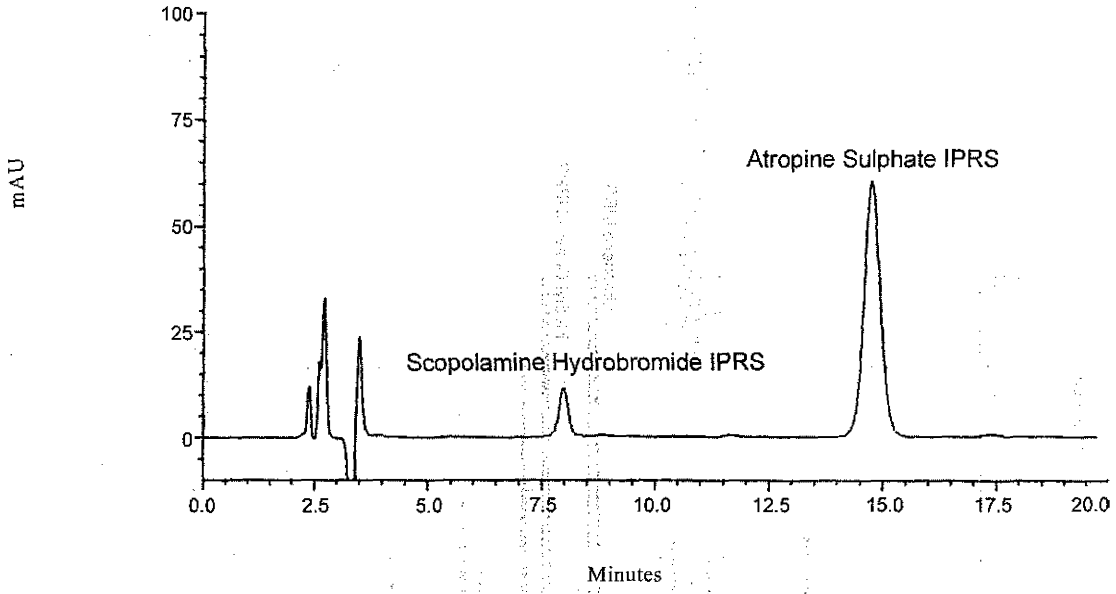
HPTLC Chromatogram of Bala

Basil Oil

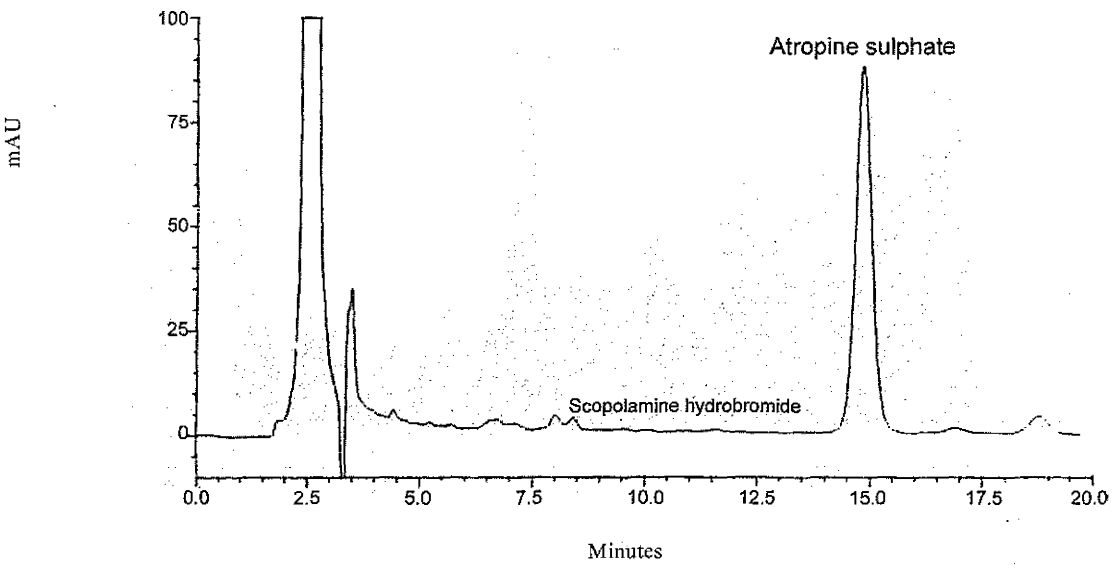


Gas Chromatogram of Basil Oil IPRS

Belladonna Dry Extract

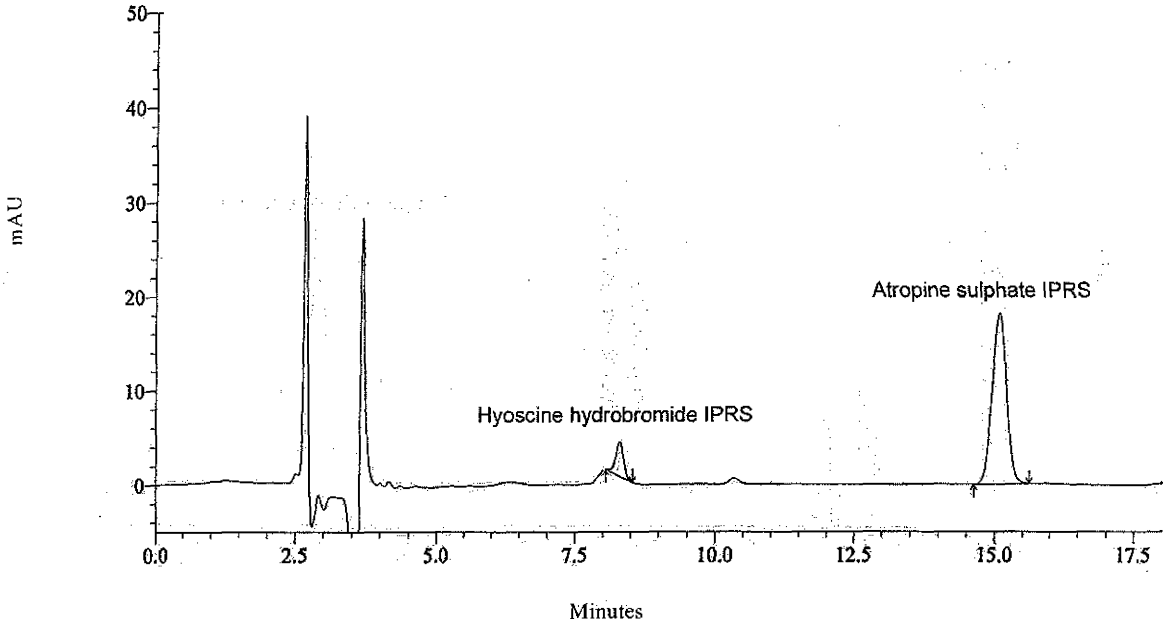


HPLC Chromatogram of atropine sulphate IPRS and scopolamine hydrobromide IPRS

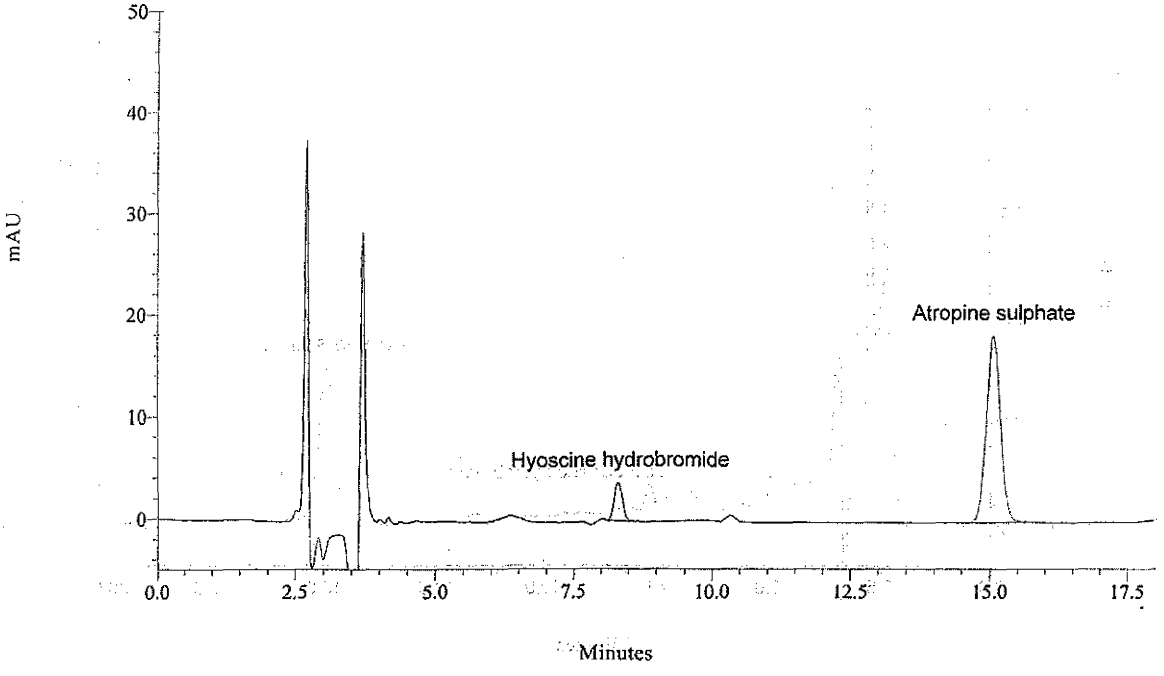


HPLC Chromatogram of Belladonna Dry Extract

Belladonna Dry Extract Tablets

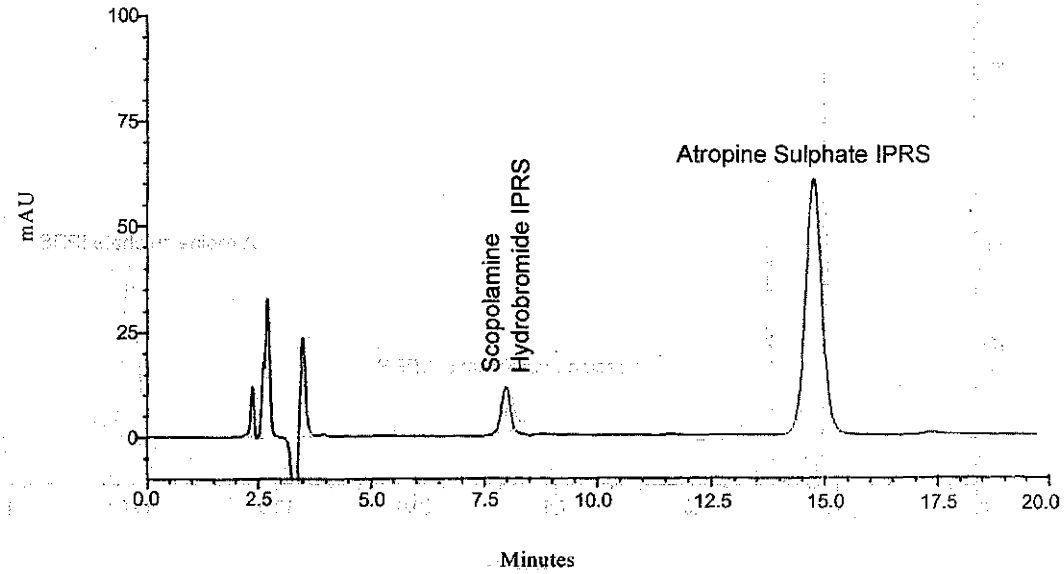


HPLC Chromatogram of atropine sulphate IPRS and scopolamine hydrobromide IPRS

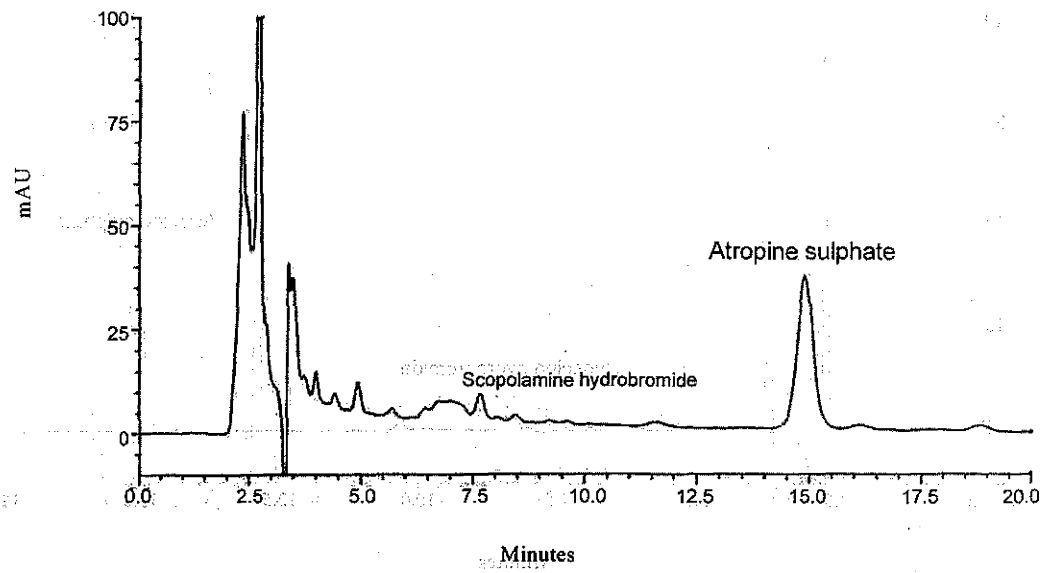


HPLC Chromatogram of Belladonna Dry Extract Tablets

Belladonna Soft Extract

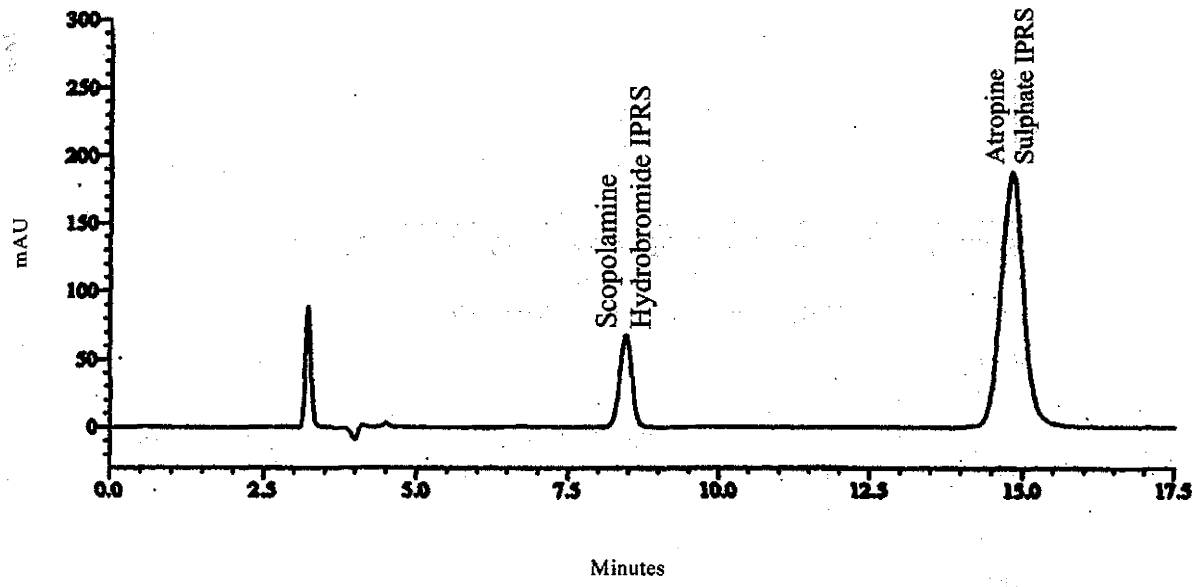


HPLC Chromatogram of atropine sulphate IPRS and scopolamine hydrobromide IPRS

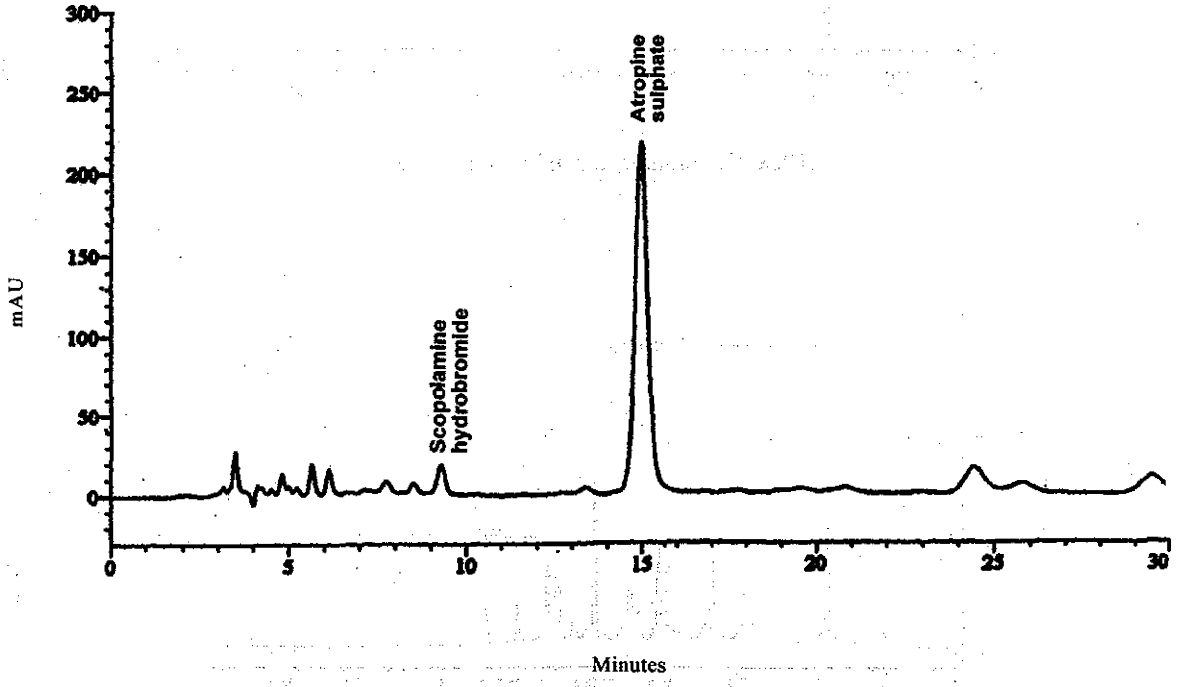


HPLC Chromatogram of Belladonna Soft Extract

Belladonna Tincture

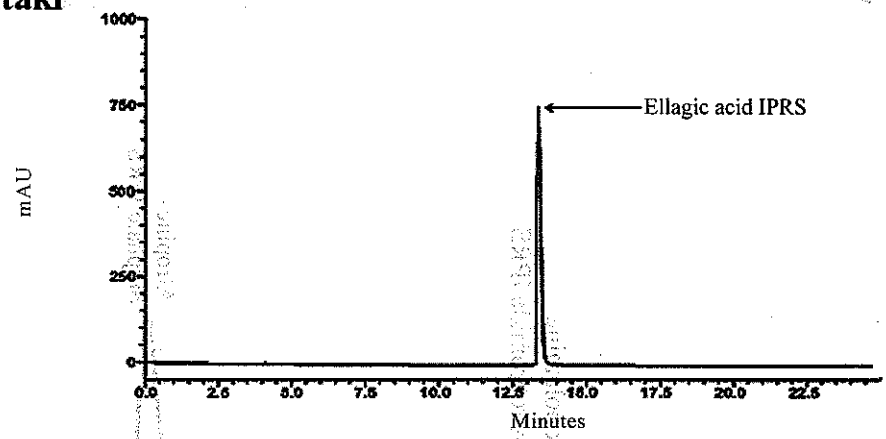


HPLC Chromatogram of scopolamine hydrobromide IPRS and atropine sulphate IPRS

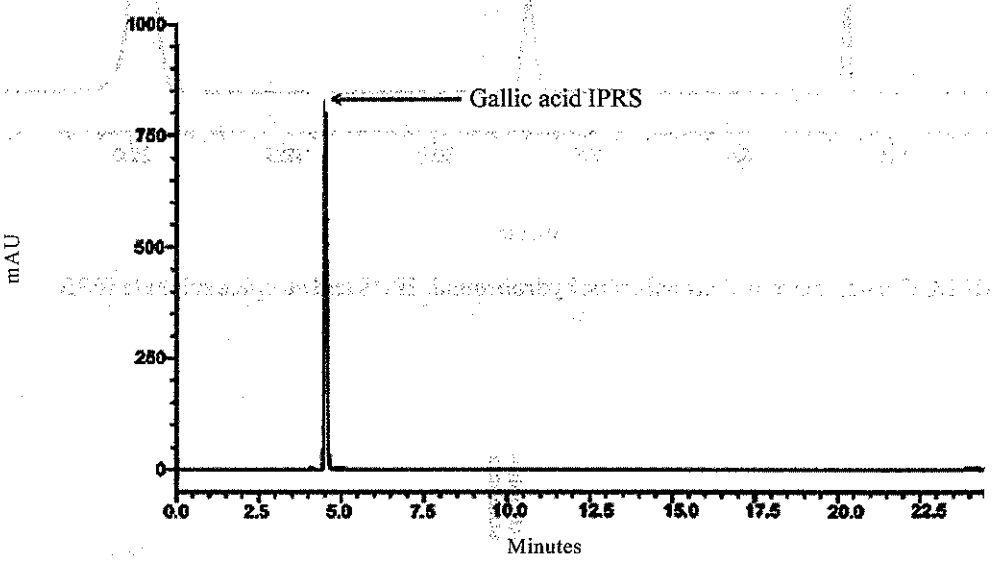


HPLC Chromatogram of Belladonna Tincture

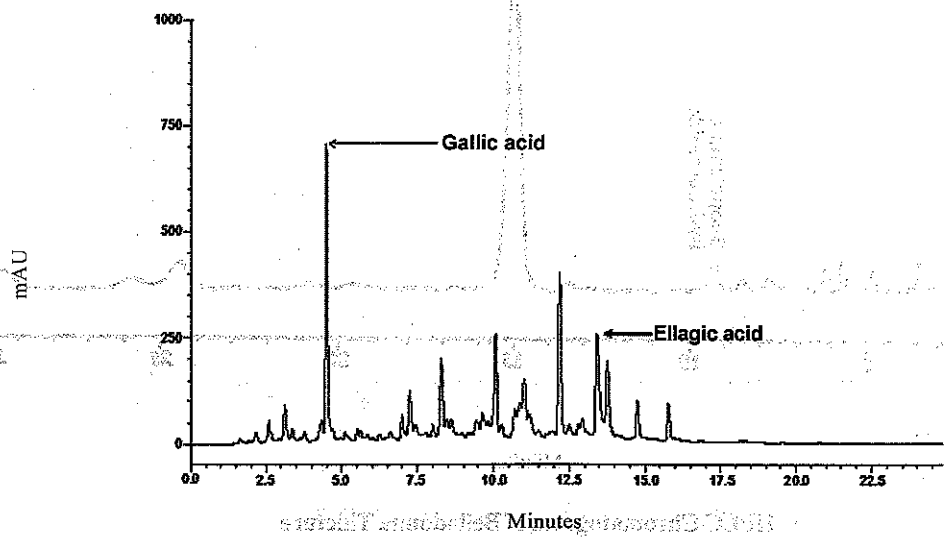
Bhibhitaki



HPLC Chromatogram of ellagic acid IPRS

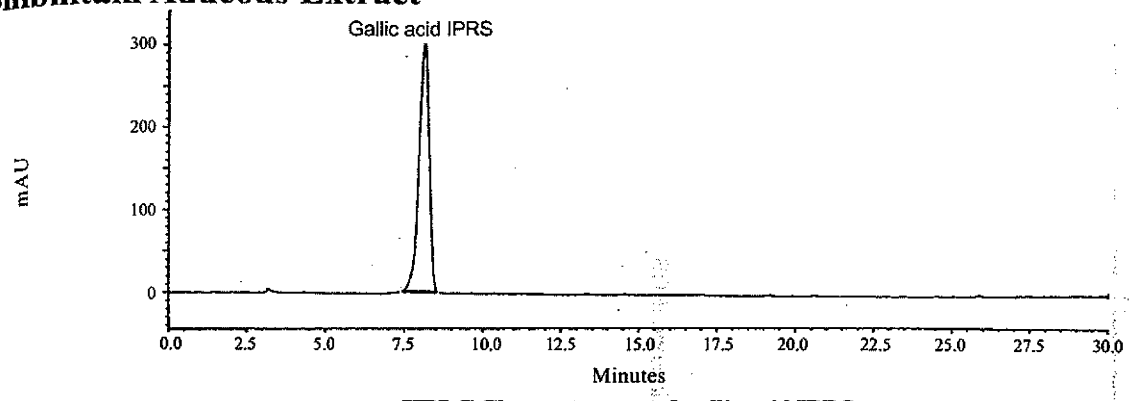


HPLC Chromatogram gallic acid IPRS

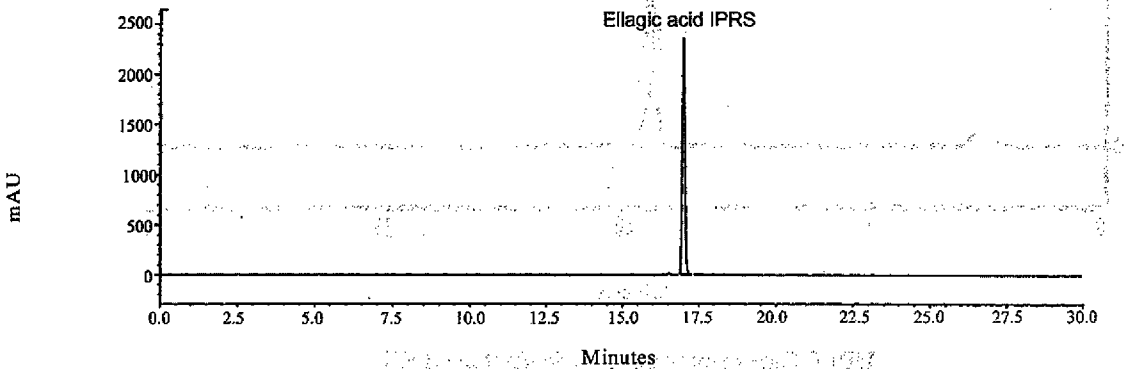


HPLC Chromatogram of Bhibhitaki

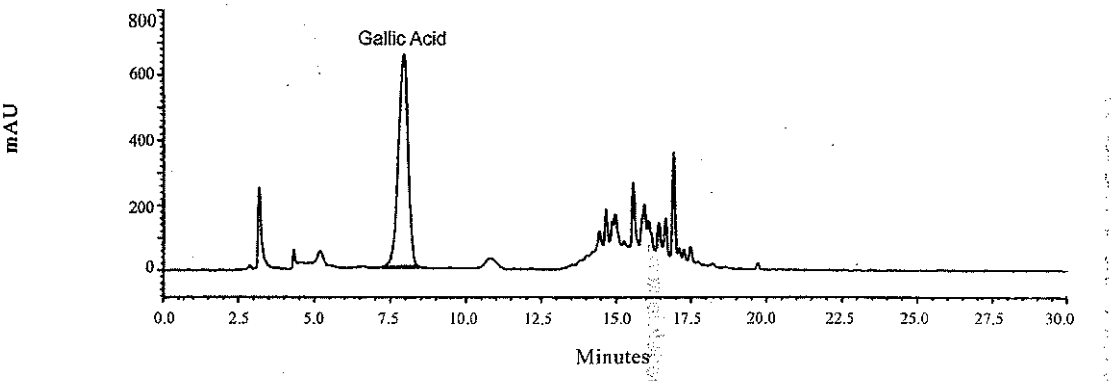
Bhibhitaki Aqueous Extract



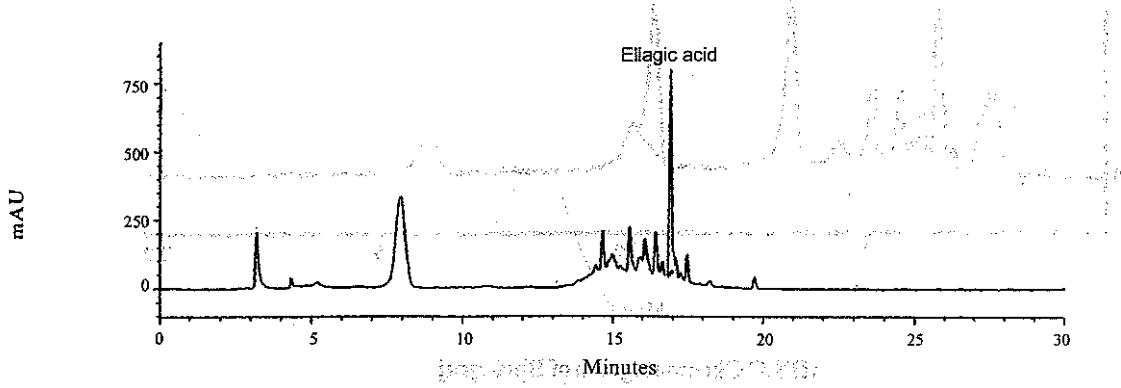
HPLC Chromatogram of gallic acid IPRS



HPLC Chromatogram of ellagic acid IPRS

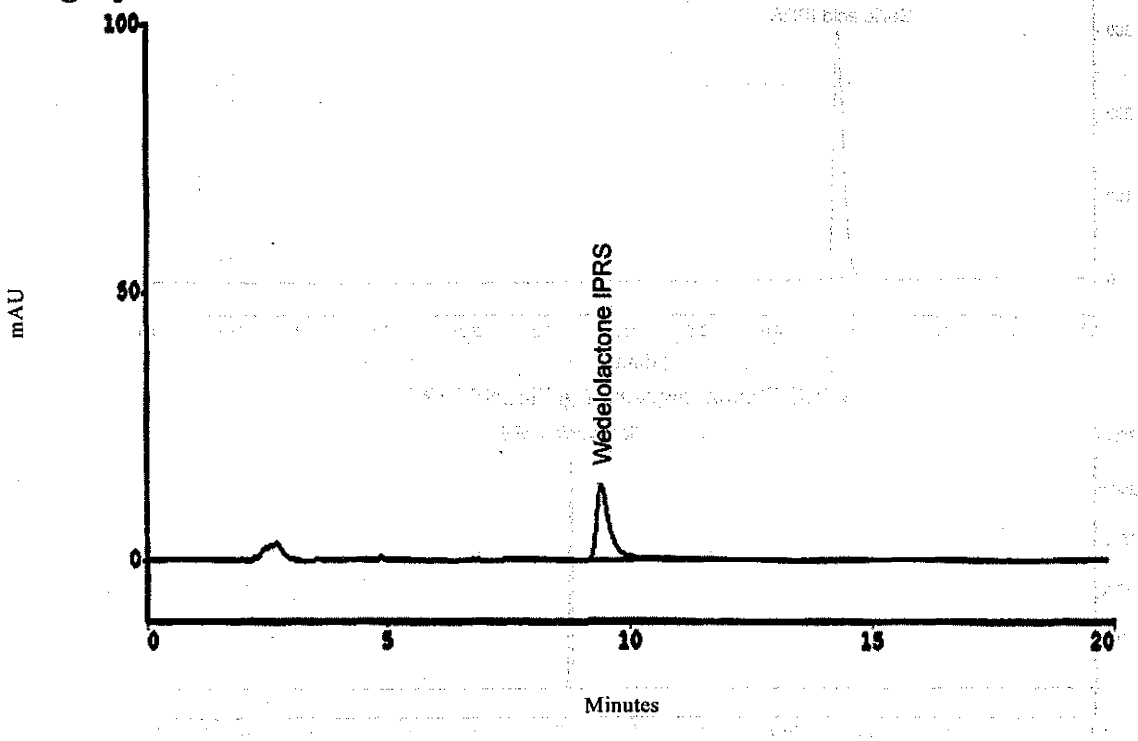


HPLC Chromatogram of Bhibhitaki Aqueous Extract

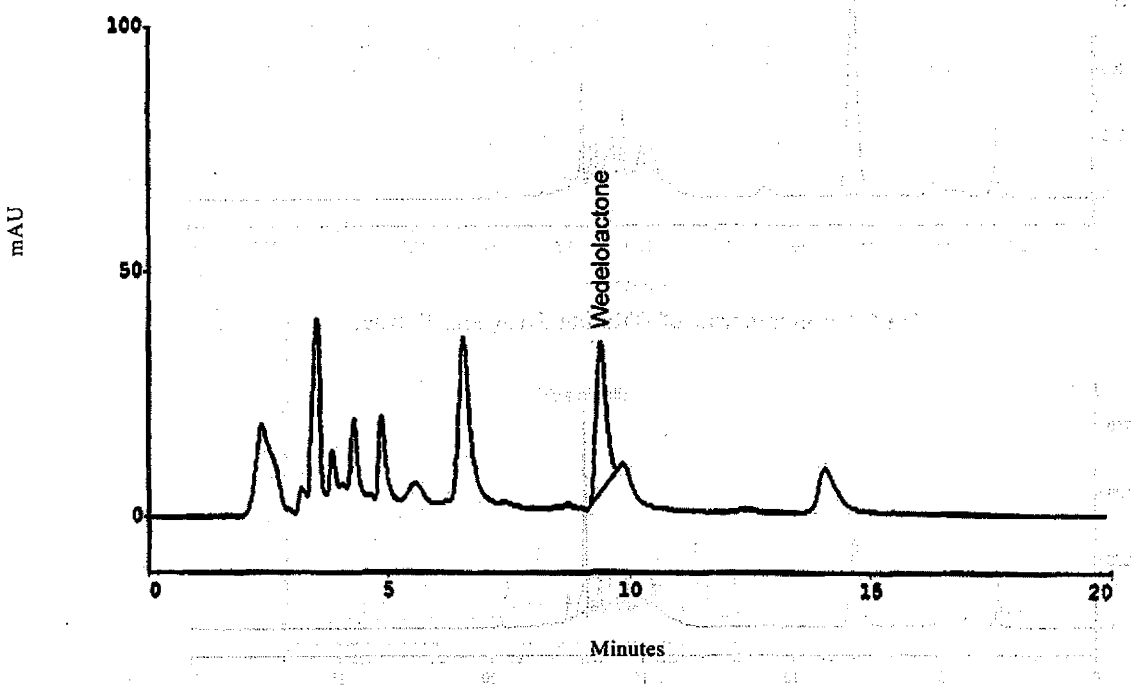


HPLC Chromatogram of Bhibhitaki Aqueous Extract

Bhringraj

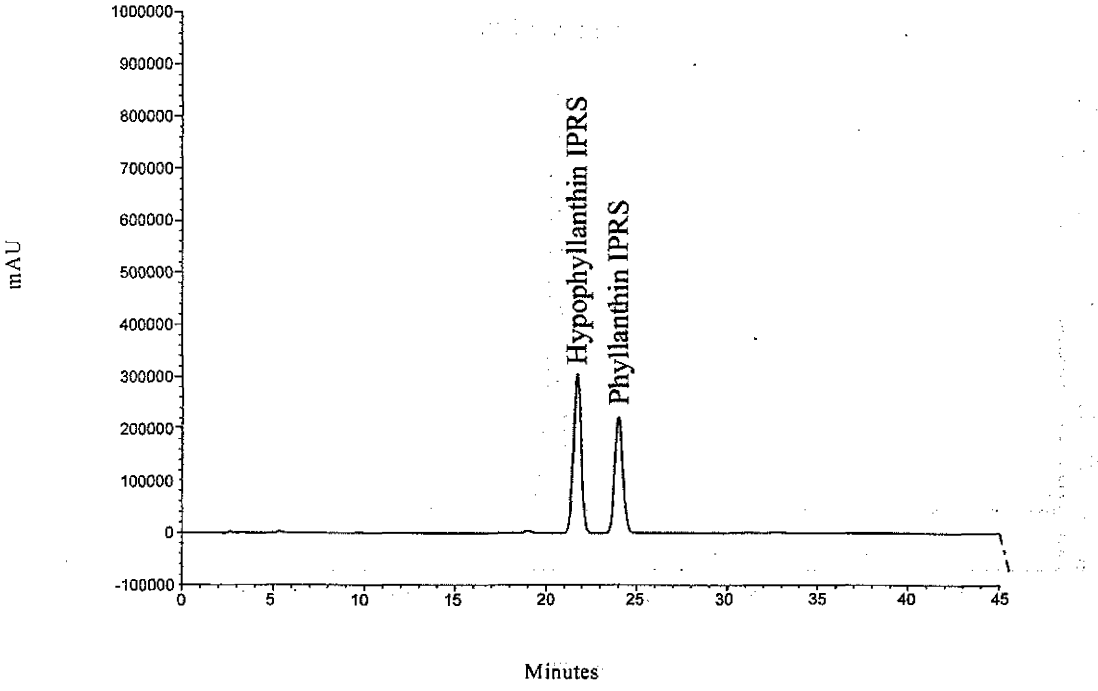


HPLC Chromatogram of wedelolactone IPRS

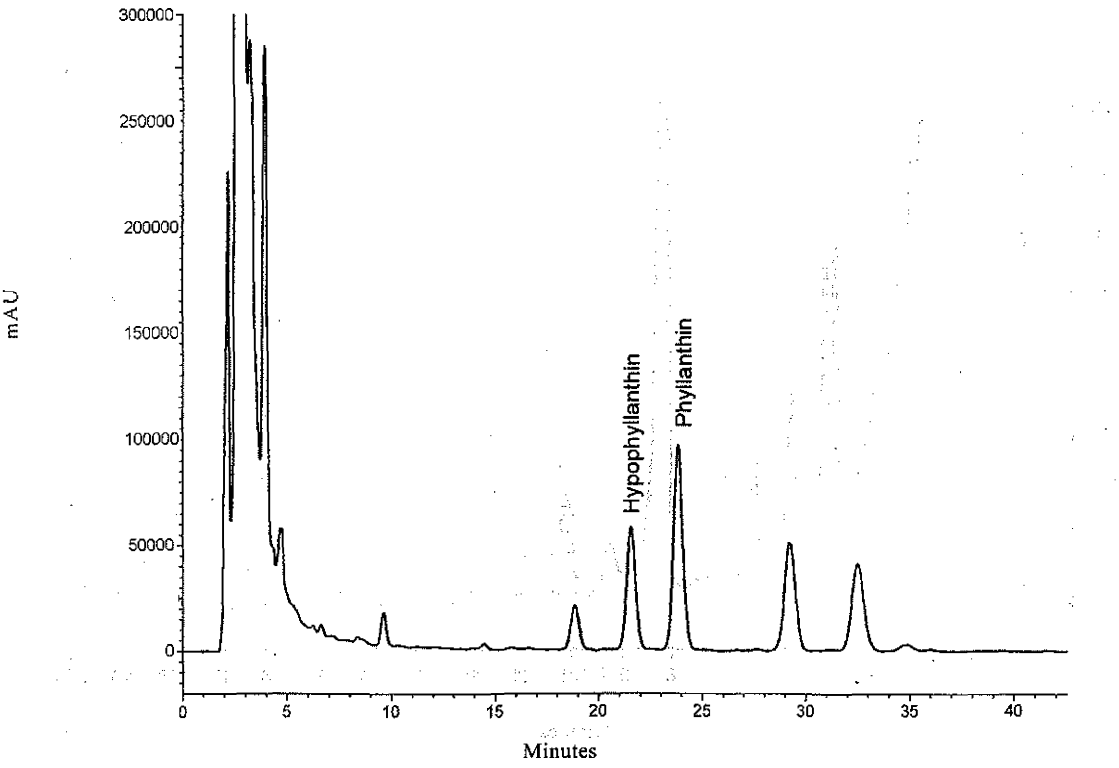


HPLC Chromatogram of Bhringraj

Bhuiamla Dry Extract

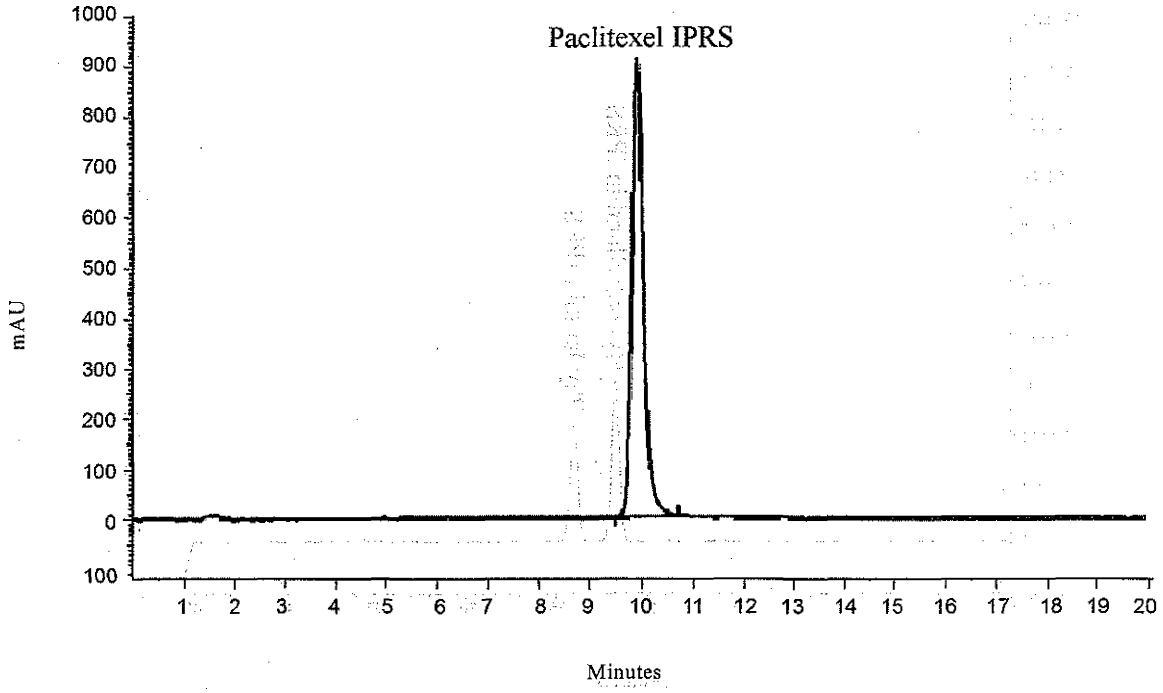


HPLC Chromatogram of hypophyllanthin IPRS and phyllanthin IPRS

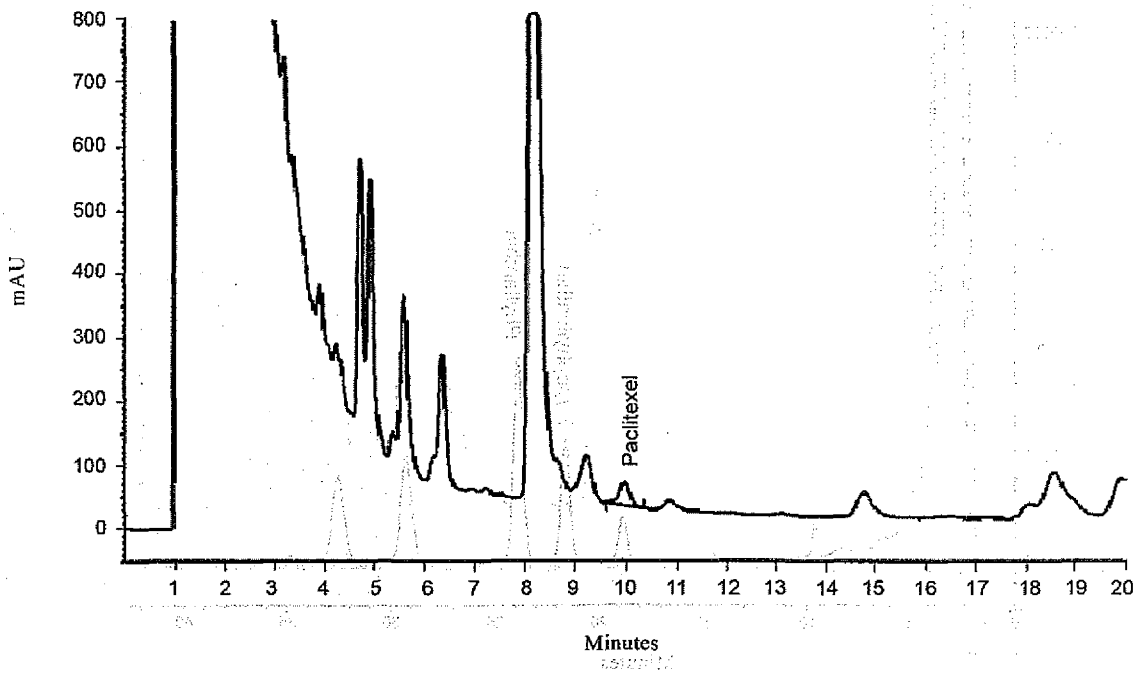


HPLC Chromatogram of Bhuiamla Dry Extract

Birmi

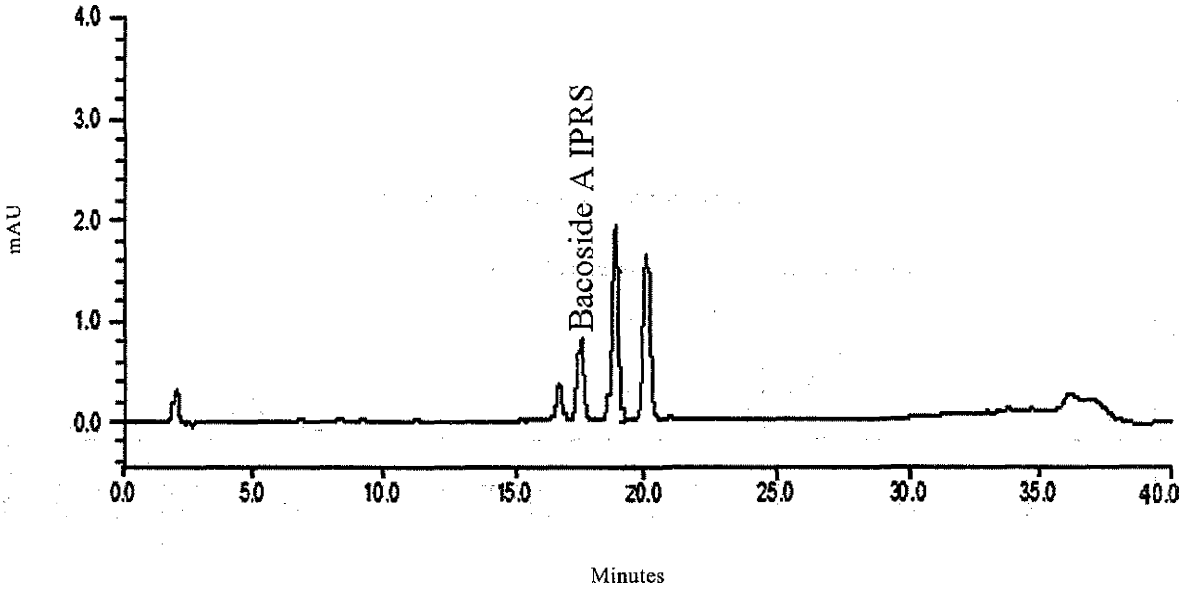


HPLC Chromatogram of paclitaxel IPRS

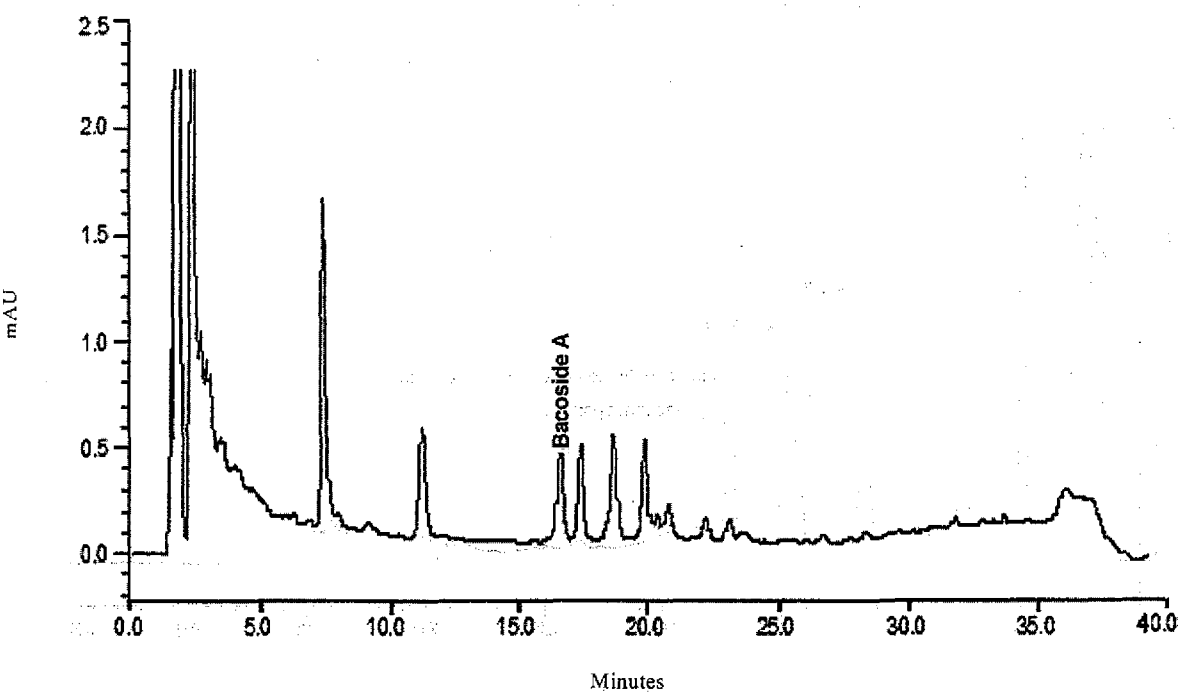


HPLC Chromatogram of Birmi

Brahmi

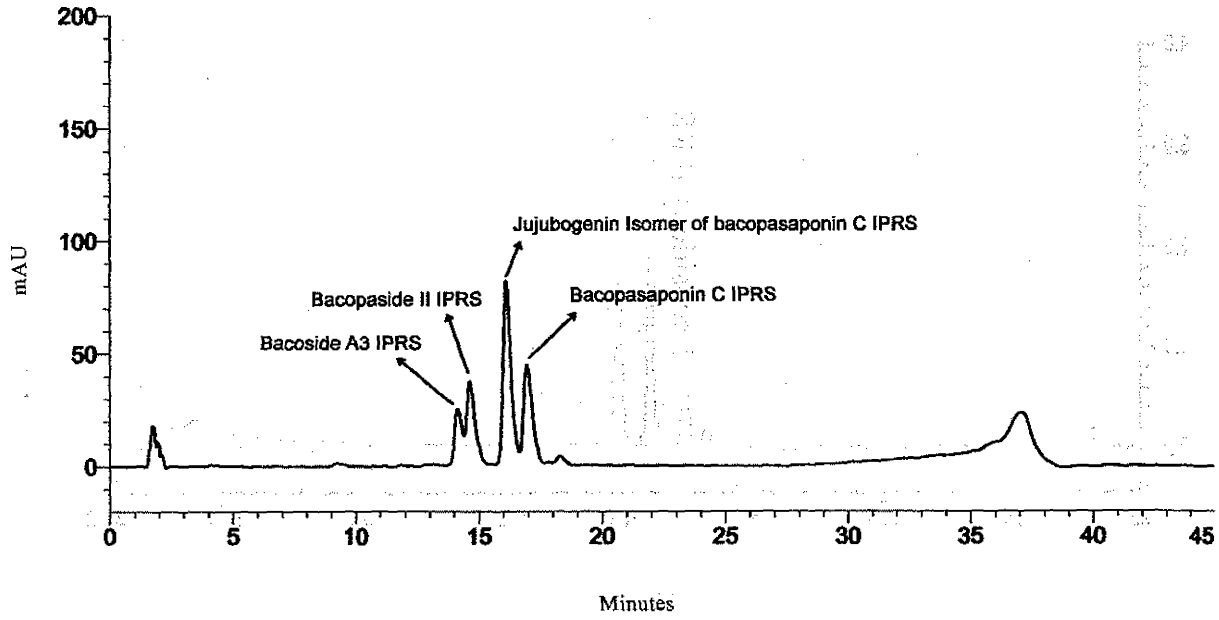


HPLC Chromatogram of bacoside A IPRS

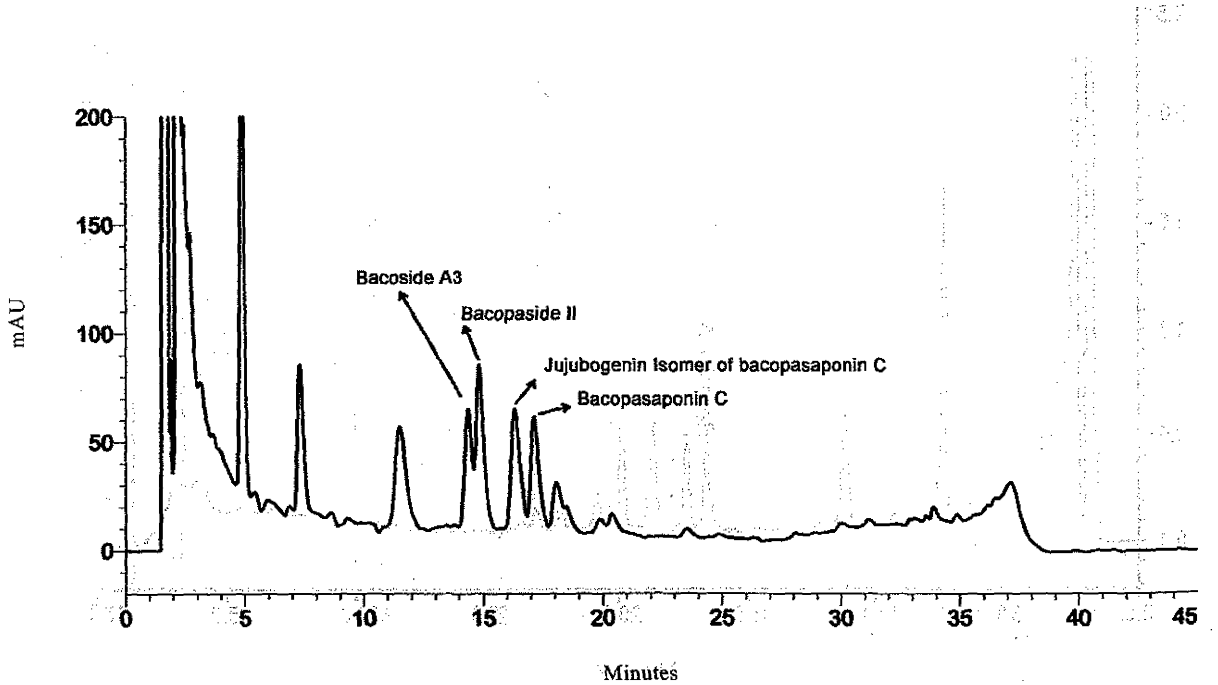


HPLC Chromatogram of Brahmi

Brahmi Extract

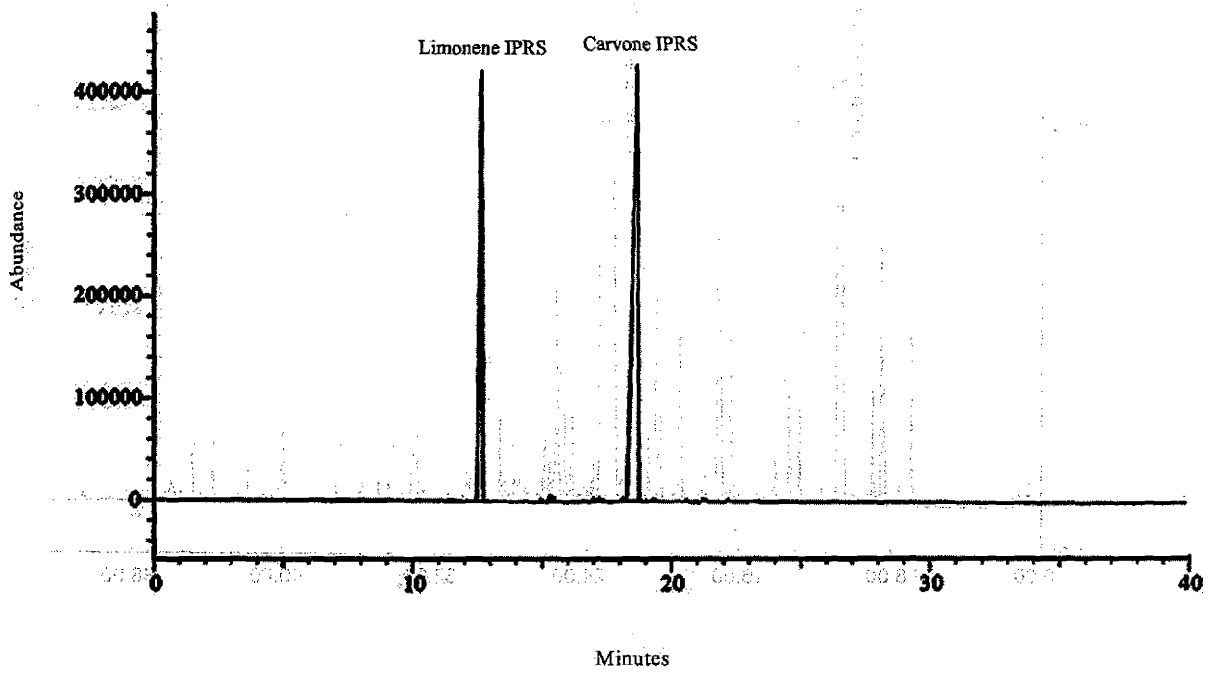


HPLC Chromatogram of bacoside A3 IPRS, bacopaside II IPRS, jujubogenin isomer of bacopasaponin C IPRS and bacopasaponin C IPRS



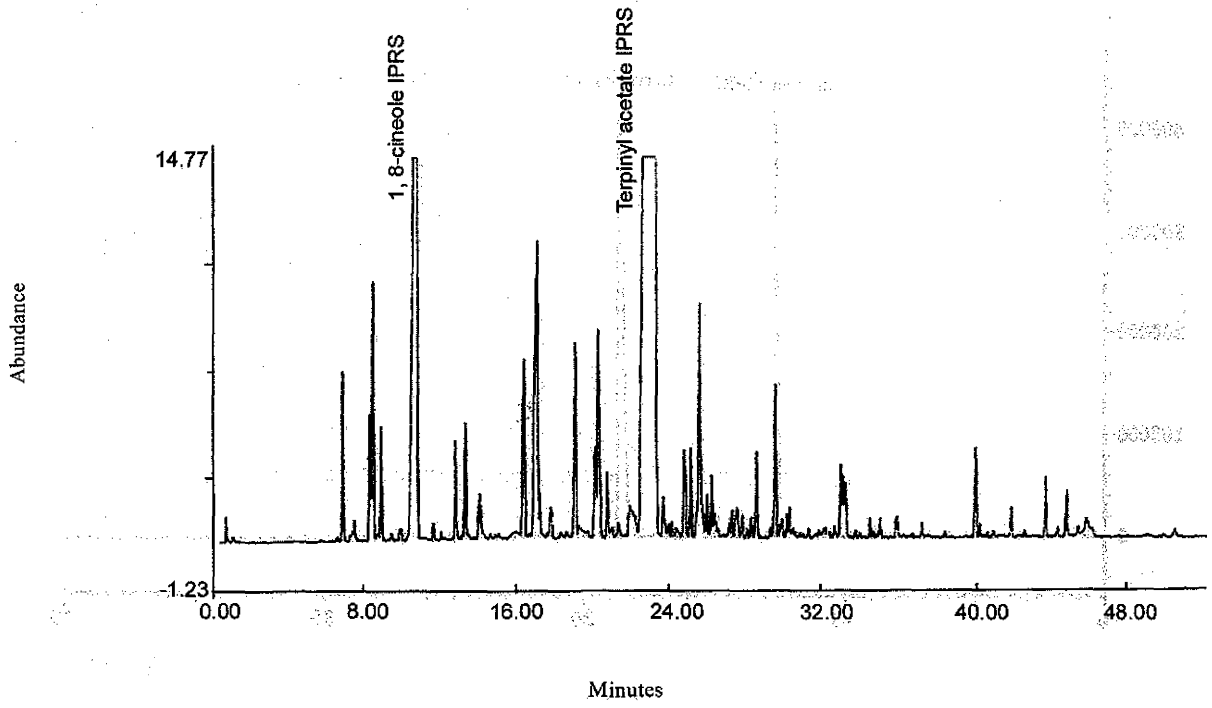
HPLC Chromatogram of Brahmi Extract

Caraway Oil



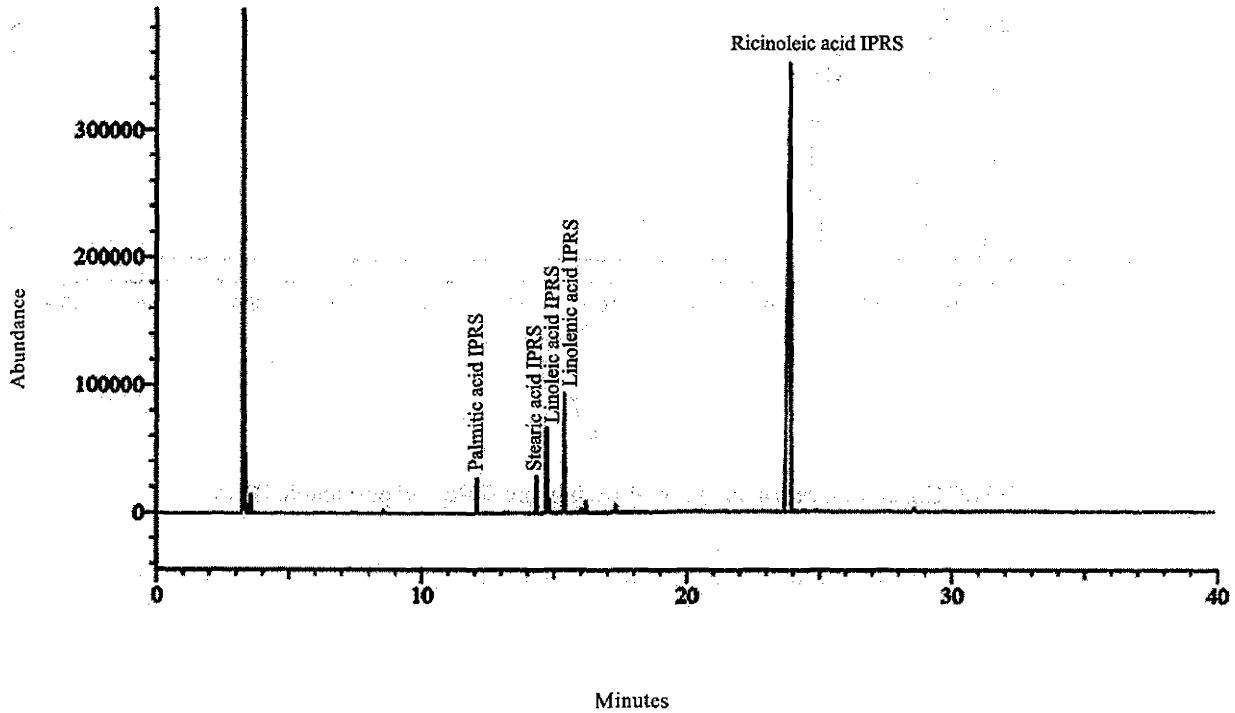
Gas Chromatogram of Caraway Oil IPRS

Cardamom Oil



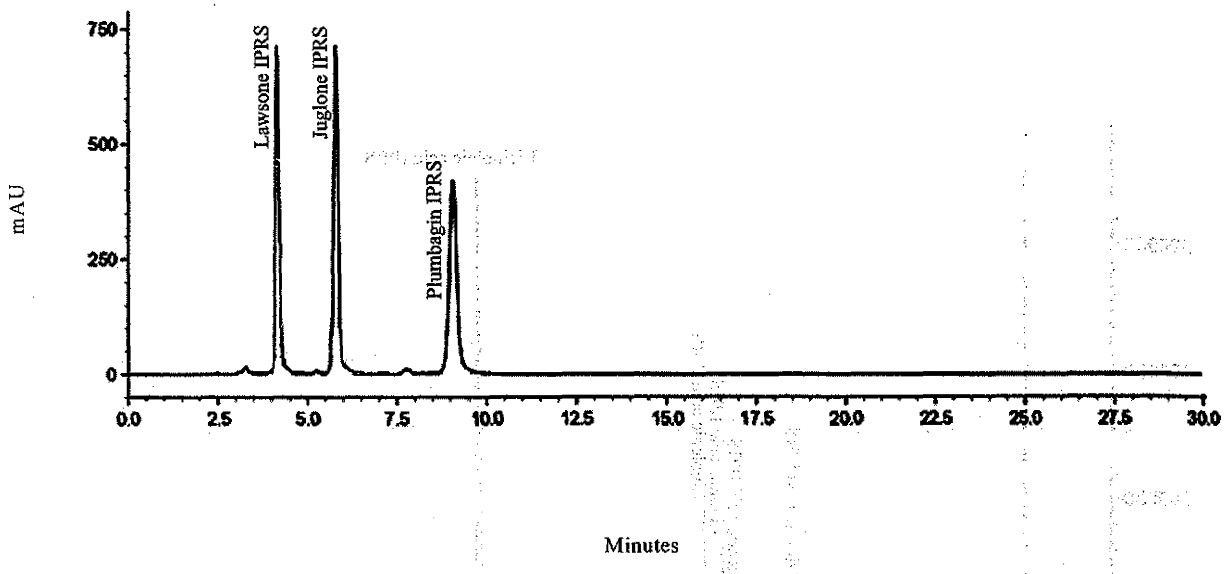
Gas Chromatogram of Cardamom Oil IPRS

Castor Oil

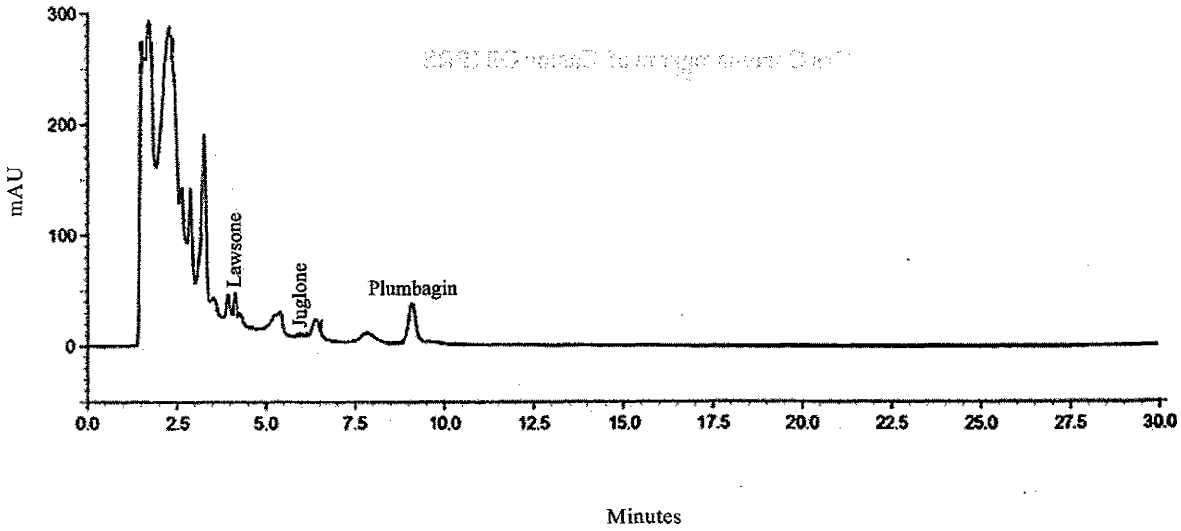


Gas Chromatogram of Castor Oil IPRS

Chitrak

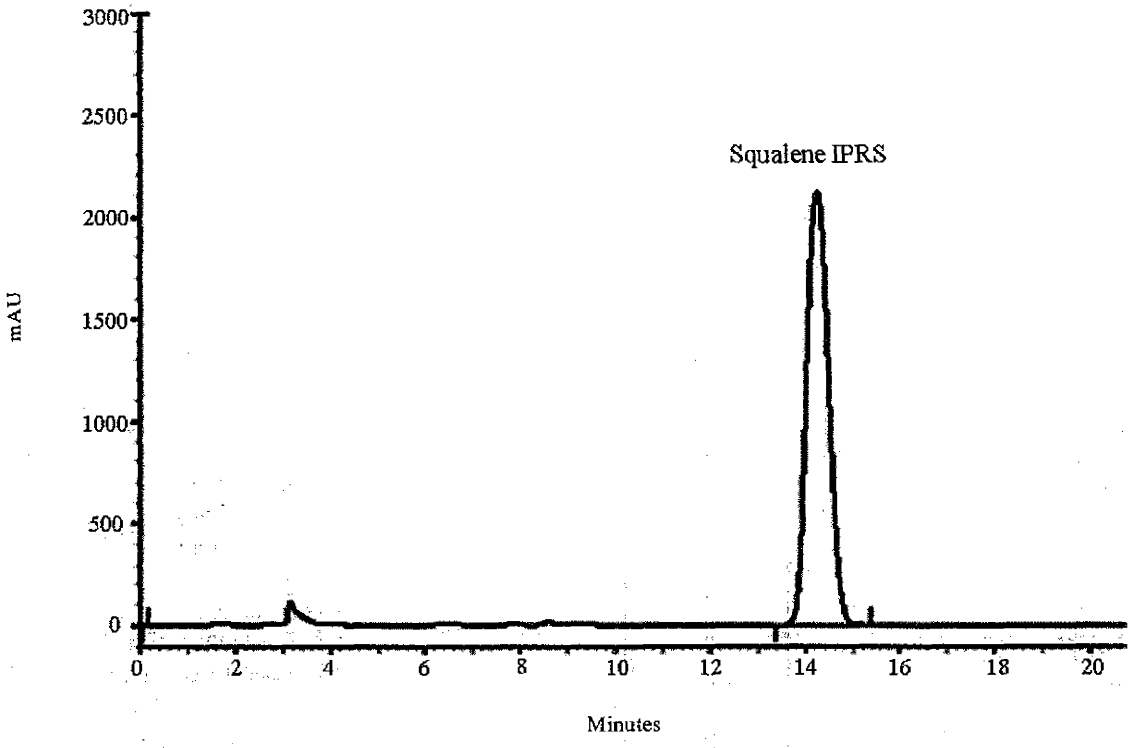


HPLC Chromatogram of lawsone IPRS, juglone IPRS and plumbagin IPRS

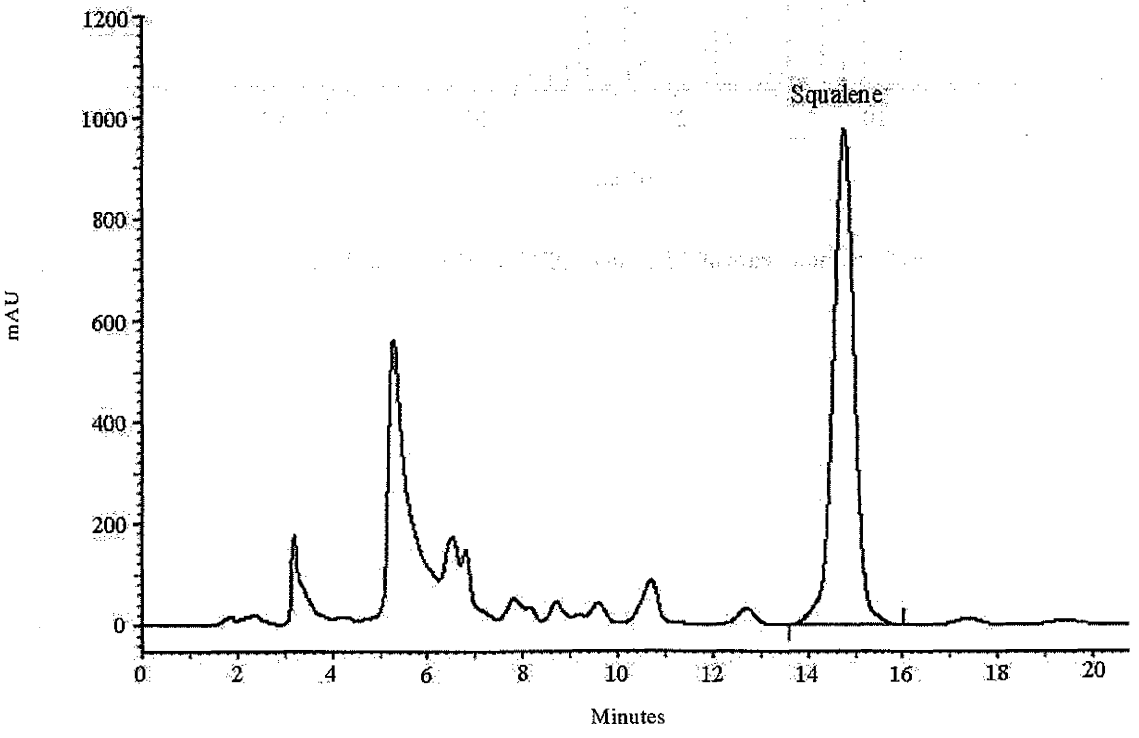


HPLC Chromatogram of Chitrak

Chaulai

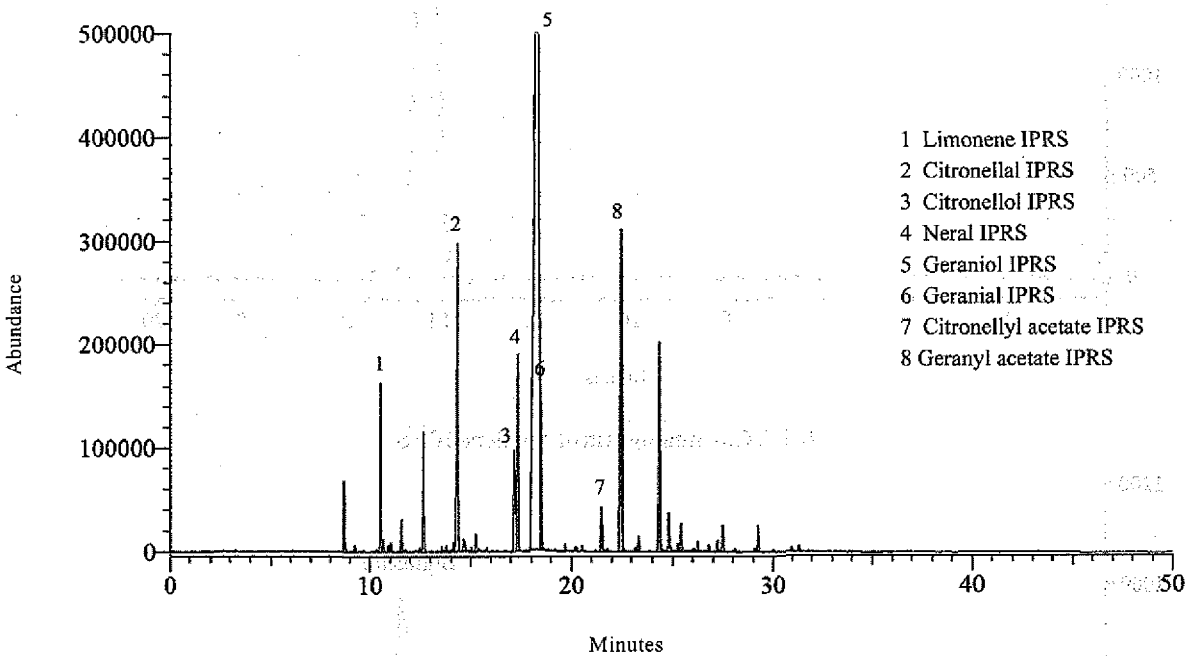


HPLC Chromatogram of squalene IPRS



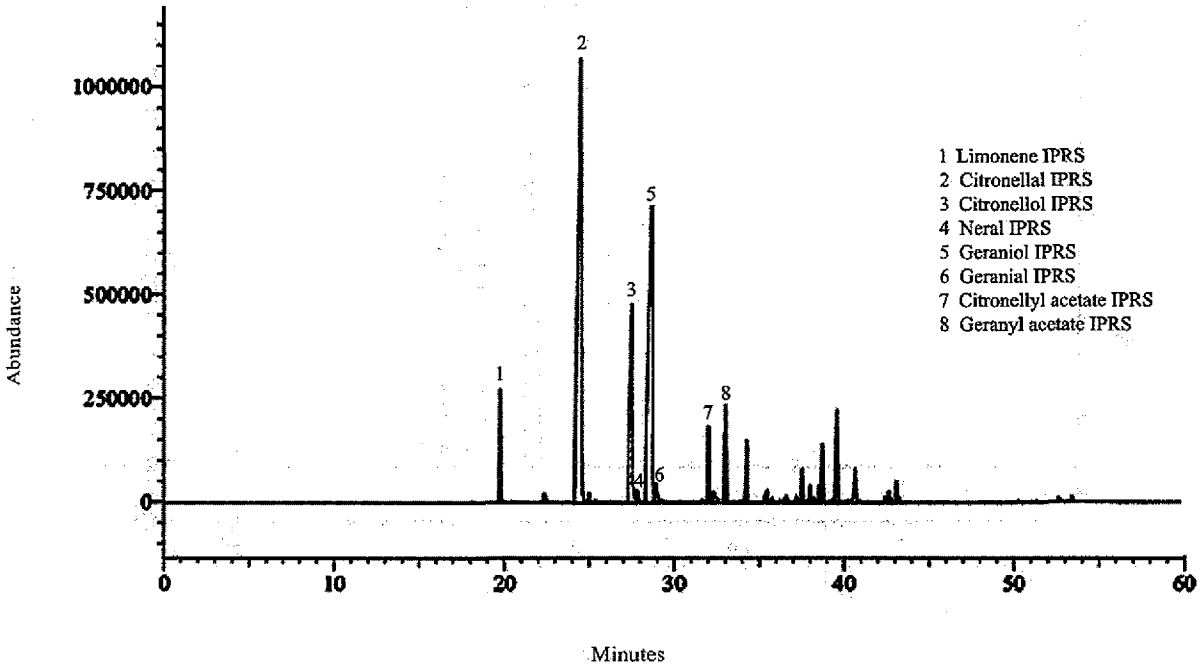
HPLC Chromatogram of Chaulai

Citronella Oil (Geraniol Type)



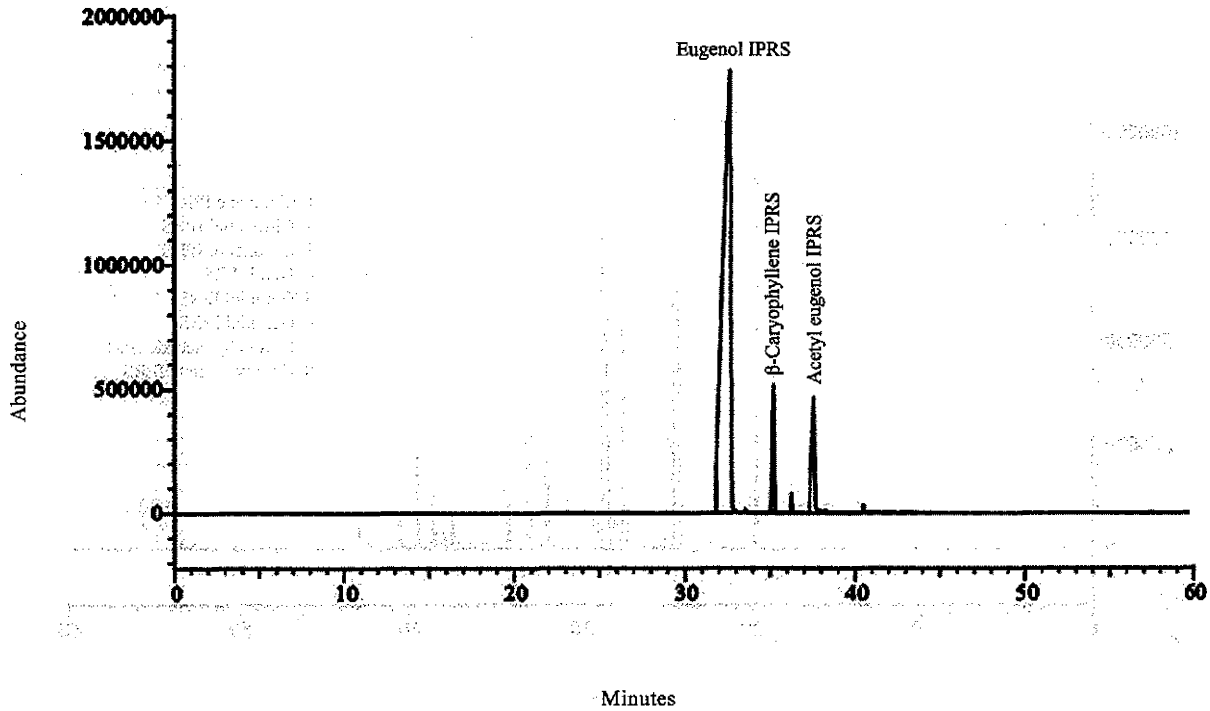
Gas Chromatogram of Citronella Oil IPRS (Geraniol Type)

Citronella Oil (Java Type)



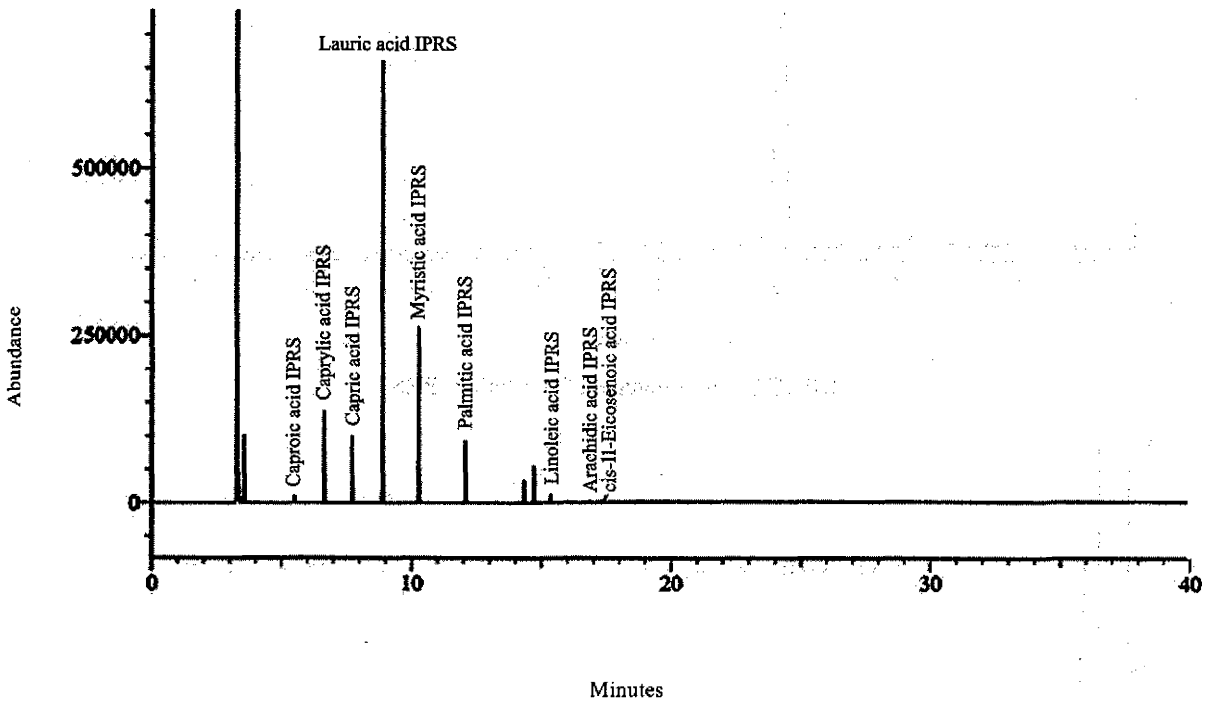
Gas Chromatogram of Citronella Oil IPRS (Java Type)

Clove Bud Oil



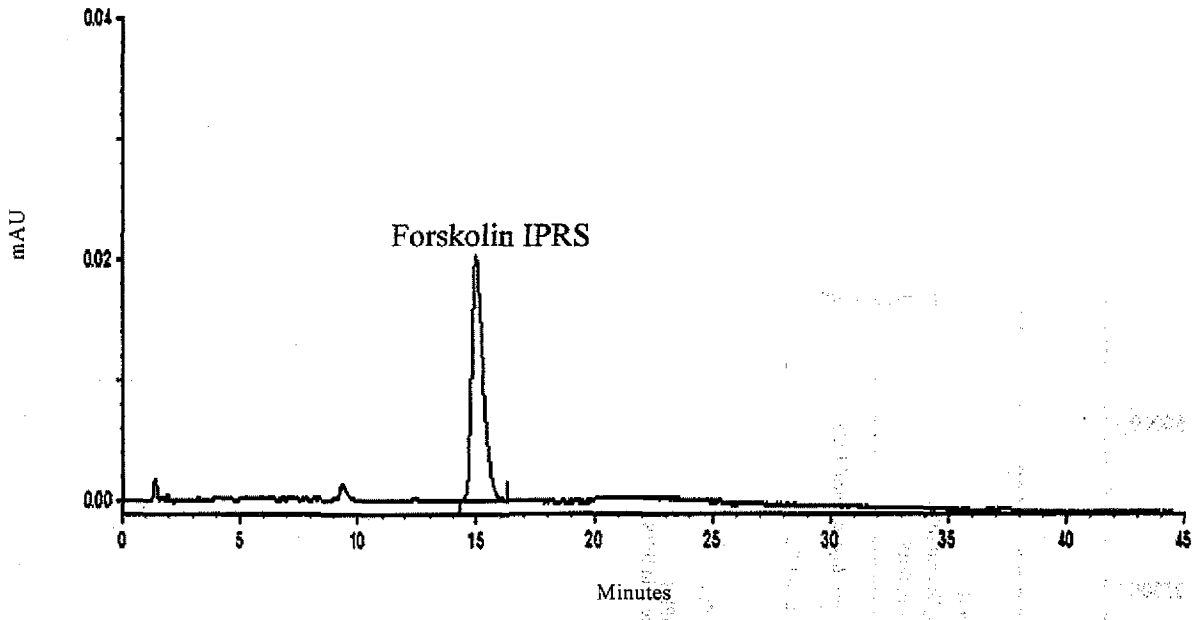
Gas Chromatogram of Clove Bud Oil IPRS

Coconut Oil

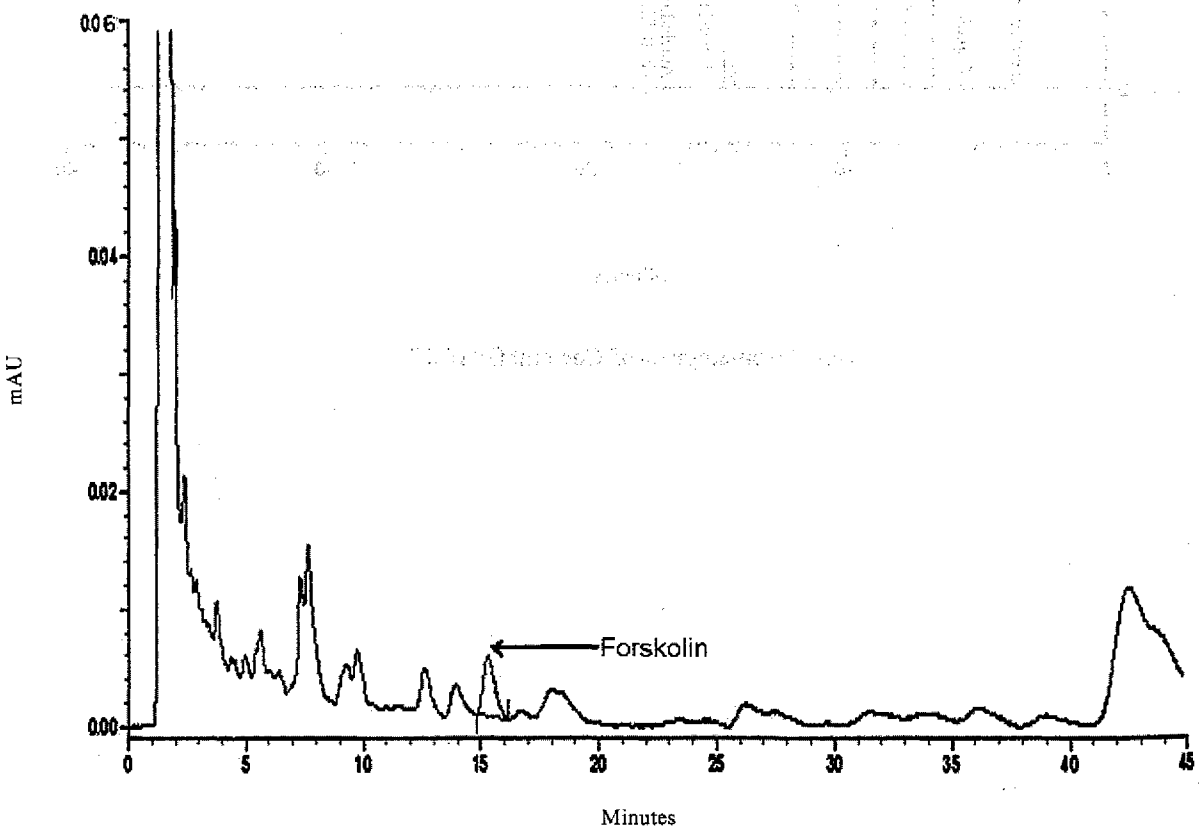


Gas Chromatogram of Coconut Oil IPRS

Coleus

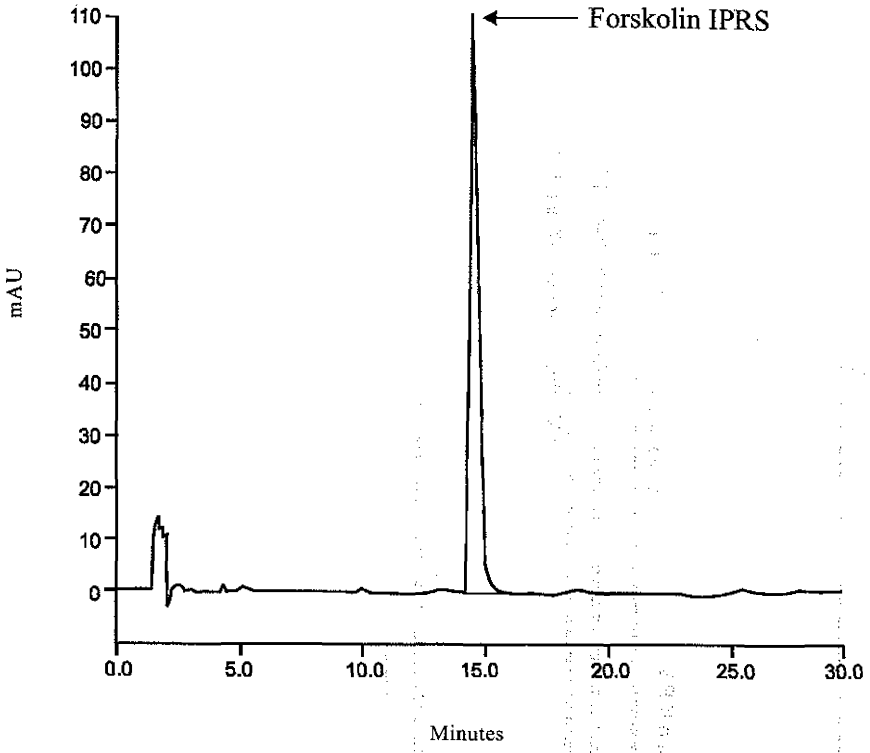


HPLC Chromatogram of forskolin IPRS

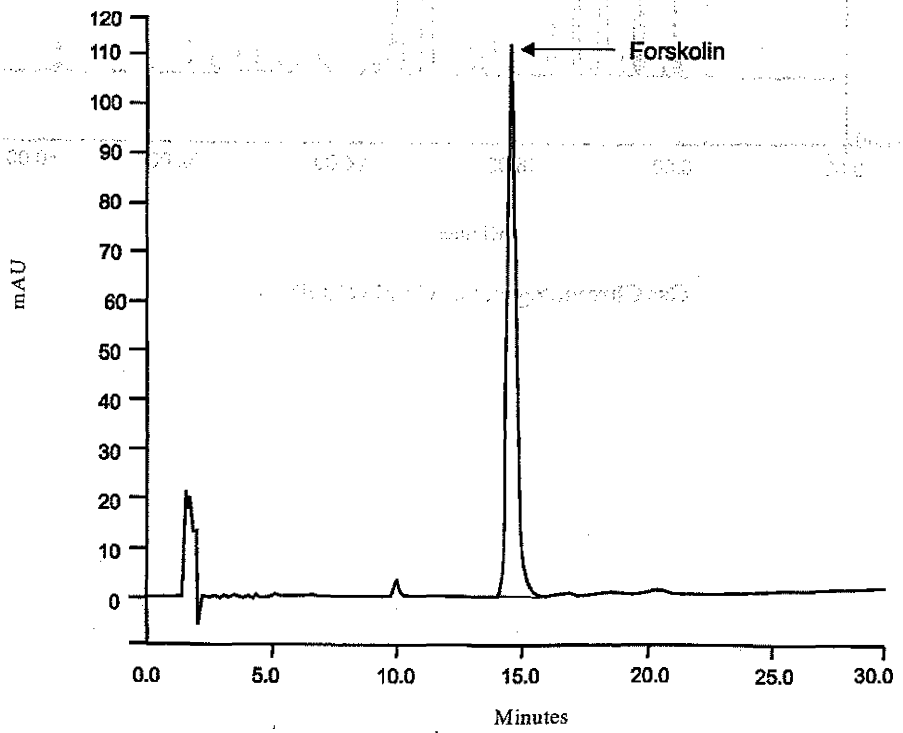


HPLC Chromatogram of Coleus

Coleus Dry Extract

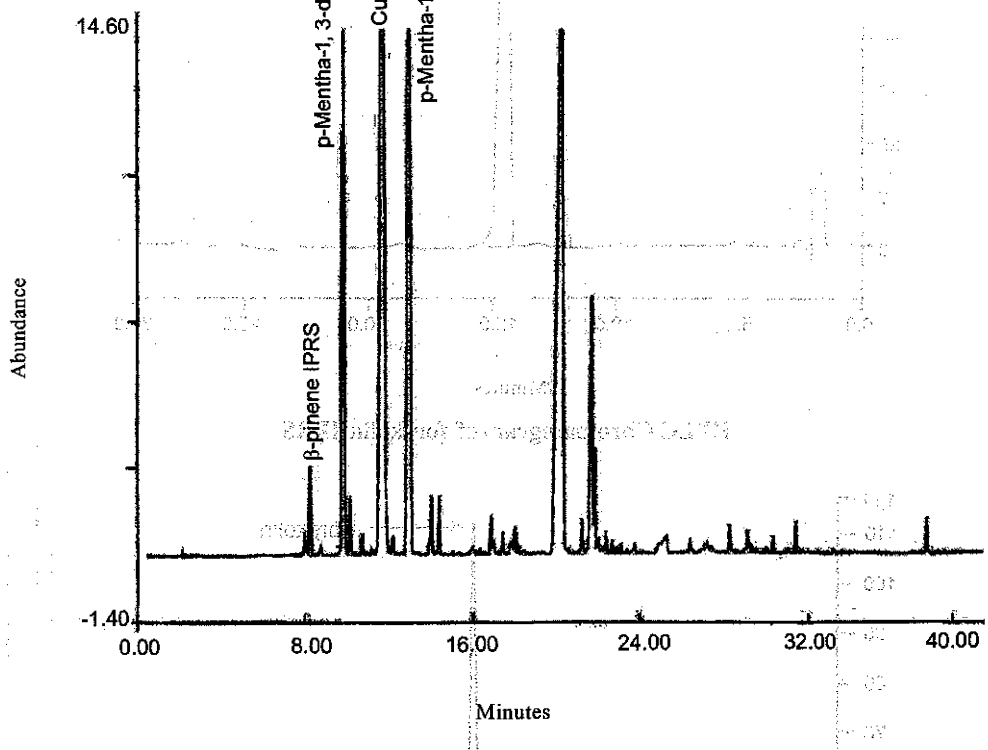


HPLC Chromatogram of forskolin IPRS



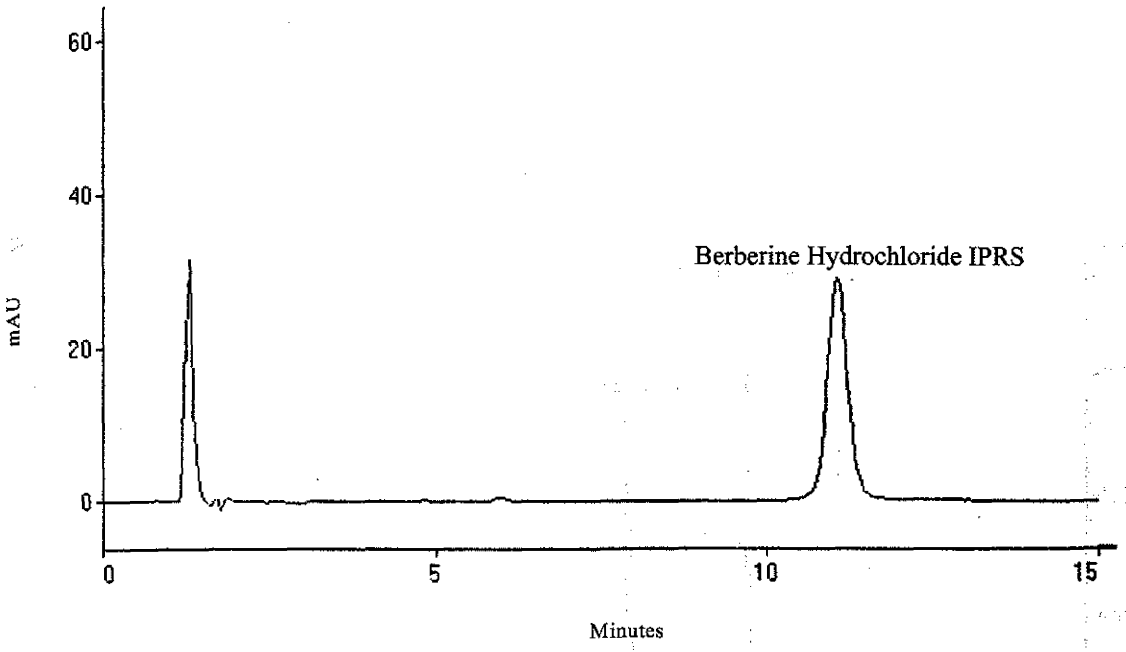
HPLC Chromatogram of Coleus Dry Extract

Cumin Oil

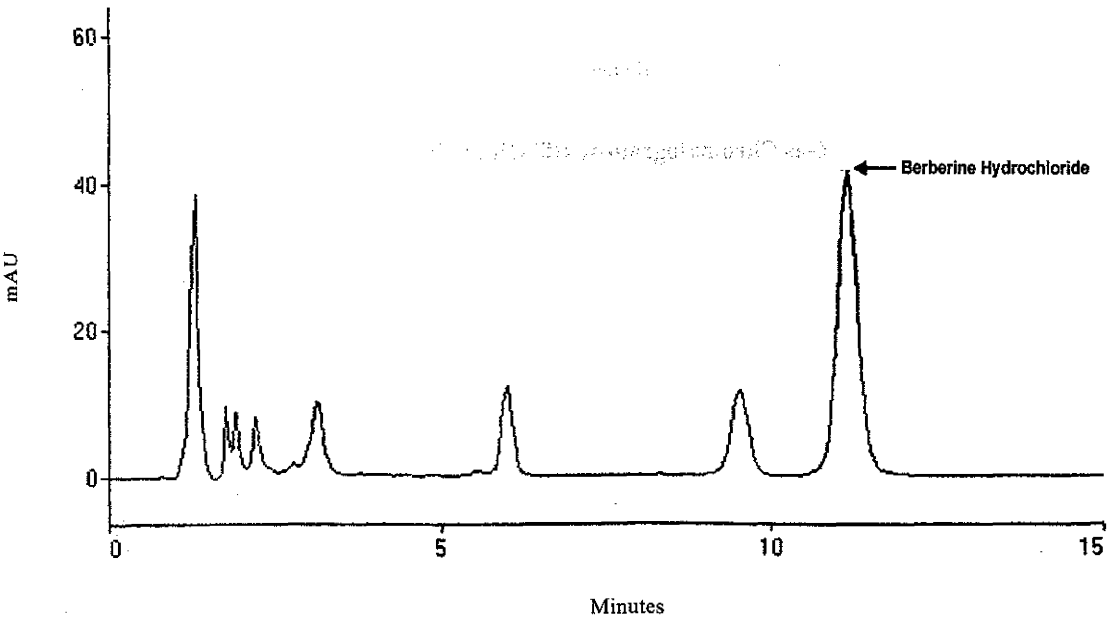


Gas Chromatogram of Cumin Oil IPRS

Daruharidra Roots and Stems

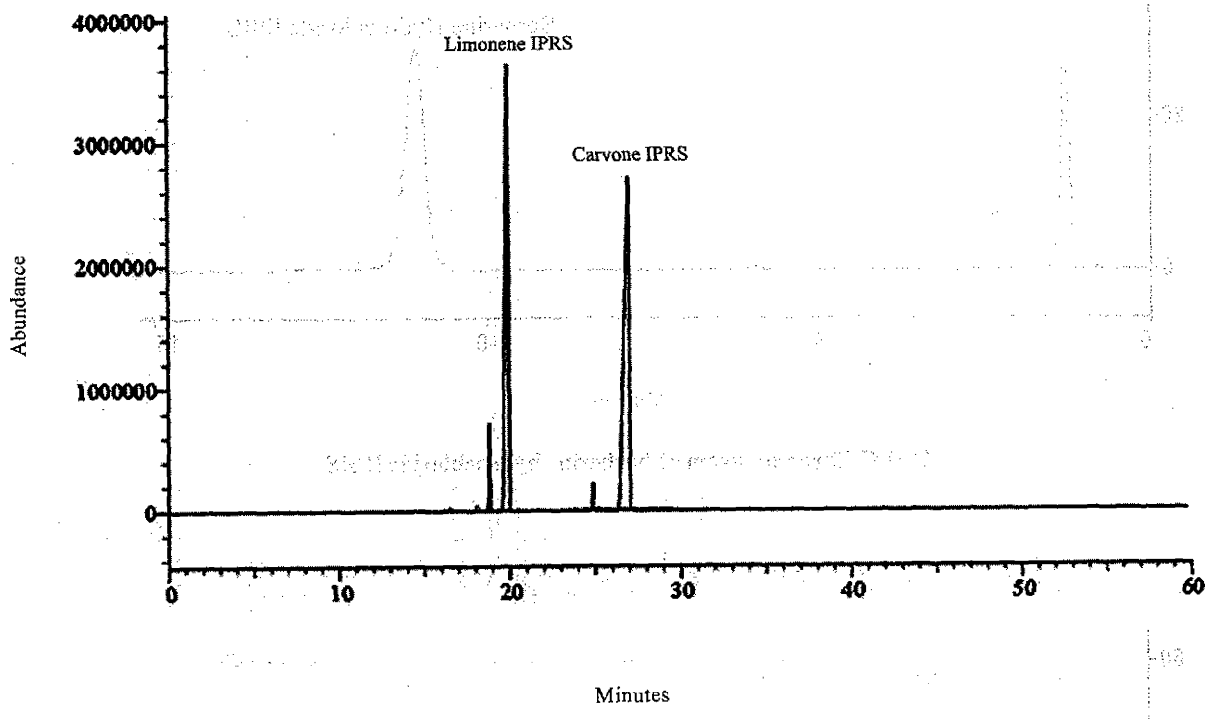


HPLC Chromatogram of berberine hydrochloride IPRS



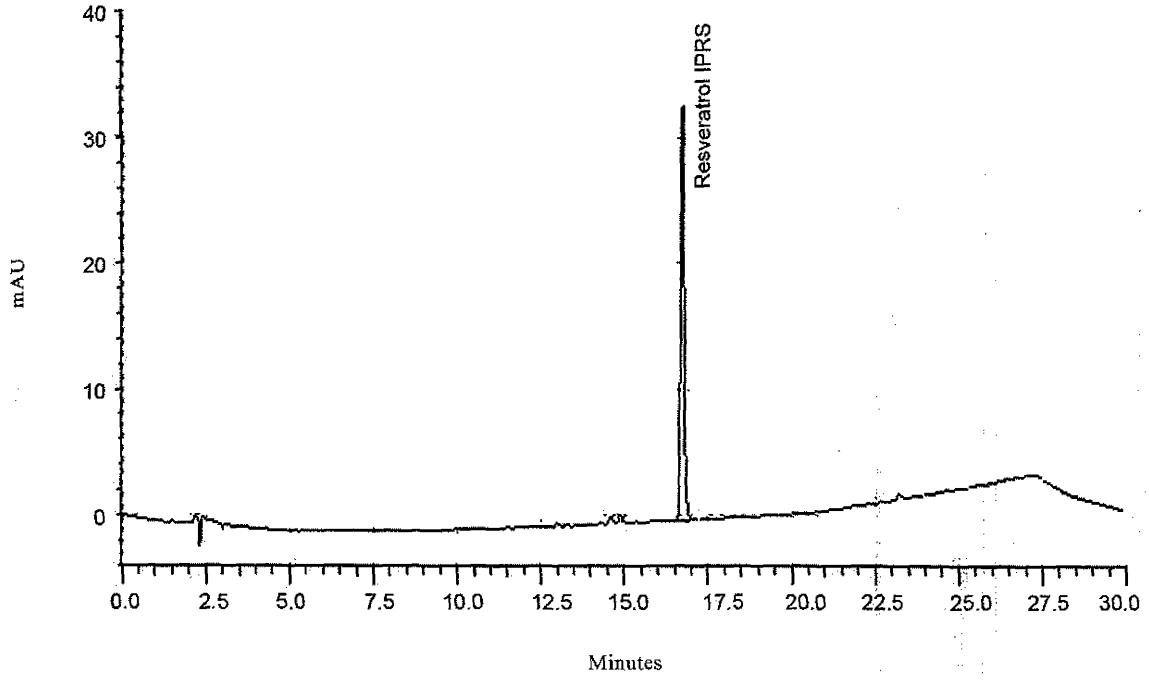
HPLC Chromatogram of Daruharidra Roots and Stems

Dill Oil

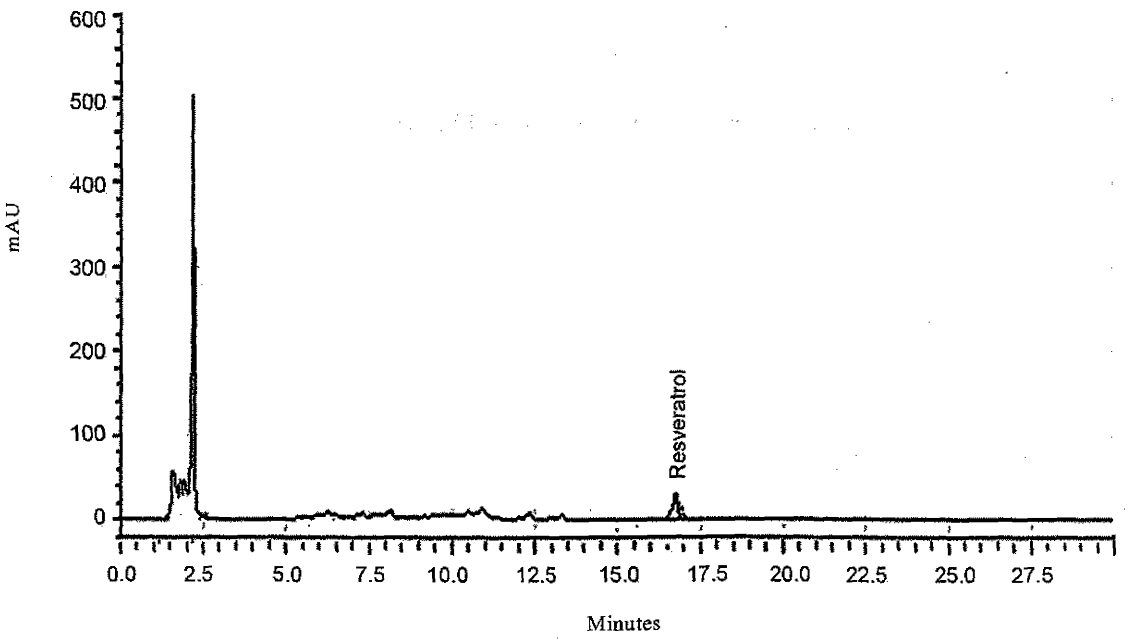


Gas Chromatogram of Dill Oil IPRS

Draksha

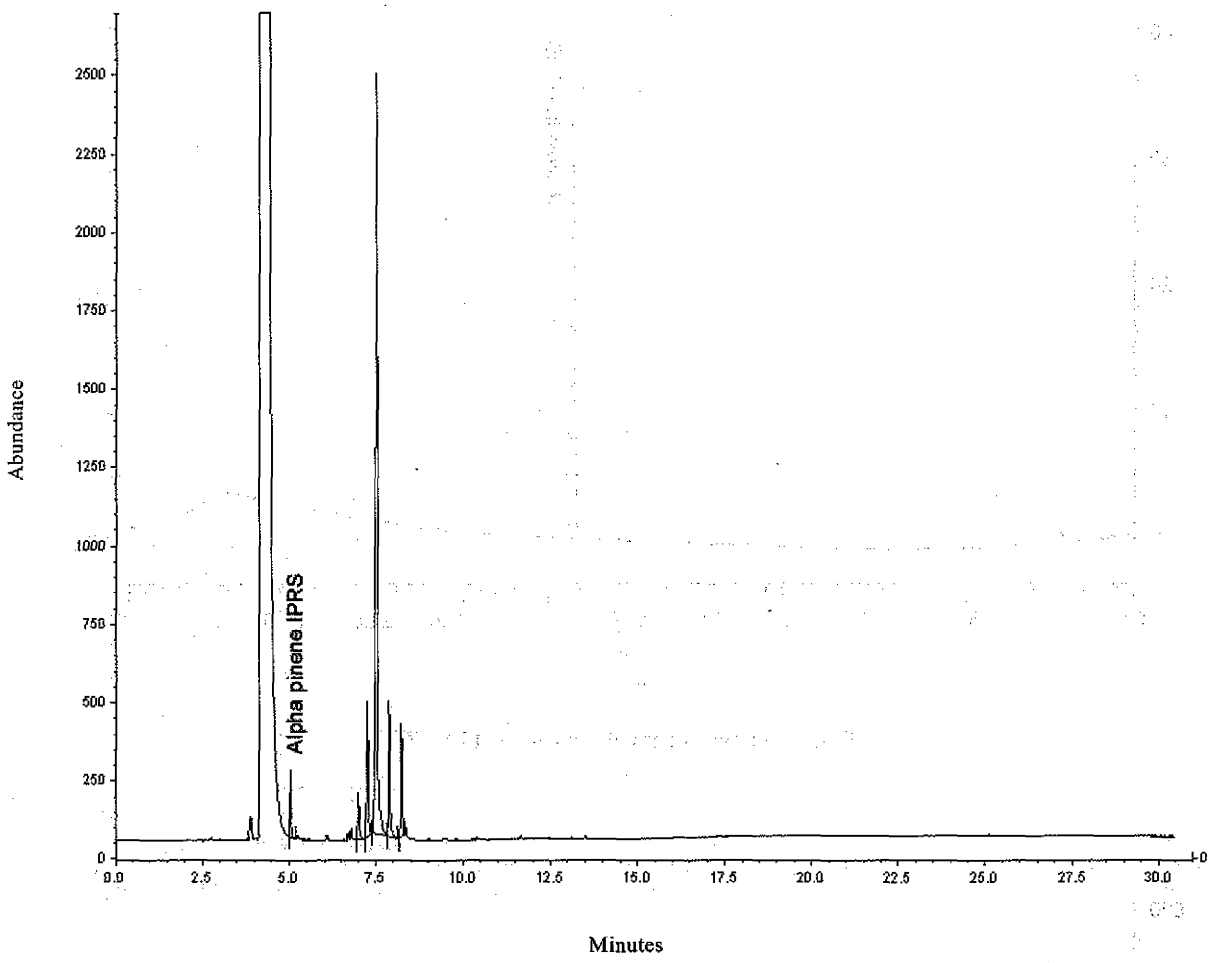


HPLC Chromatogram of resveratrol RS IPRS



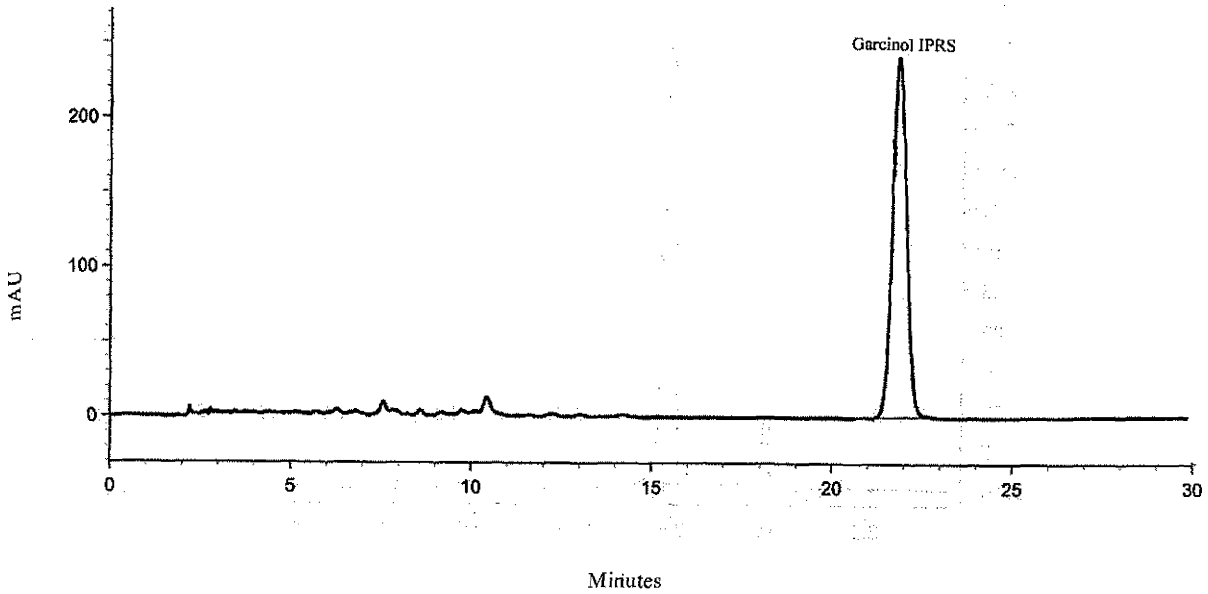
HPLC Chromatogram of Draksha

Eucalyptus Oil

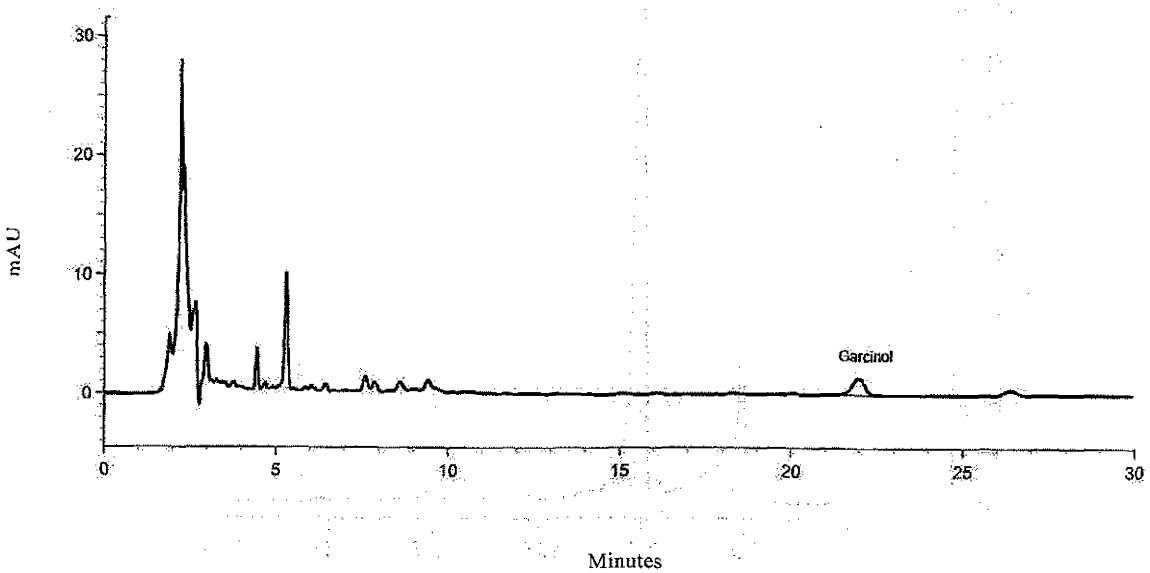


Gas Chromatogram of Eucalyptus Oil IPRS

Garcinia

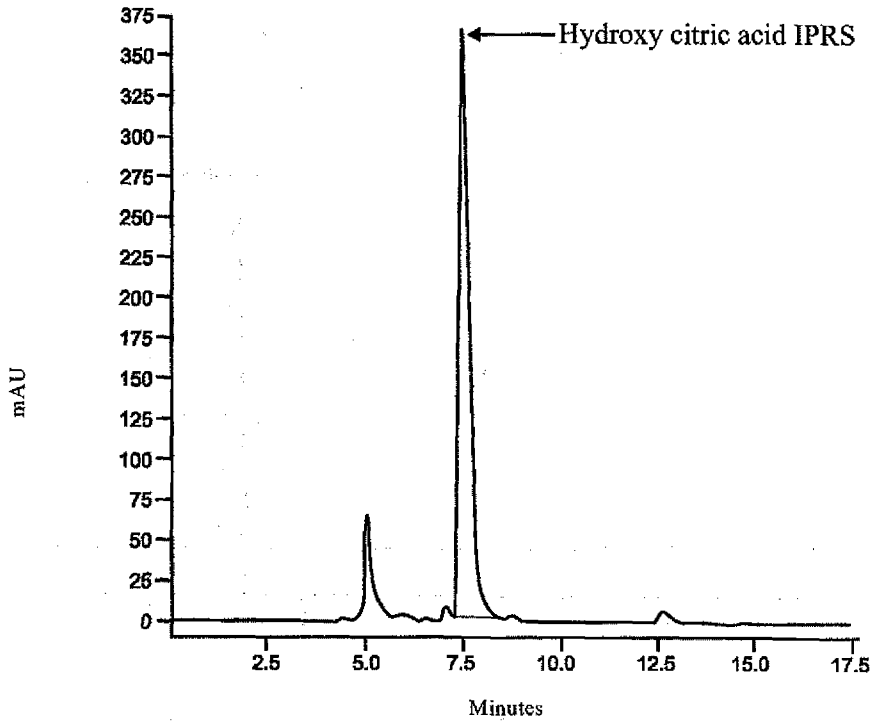


HPLC Chromatogram of Garcinol IPRS

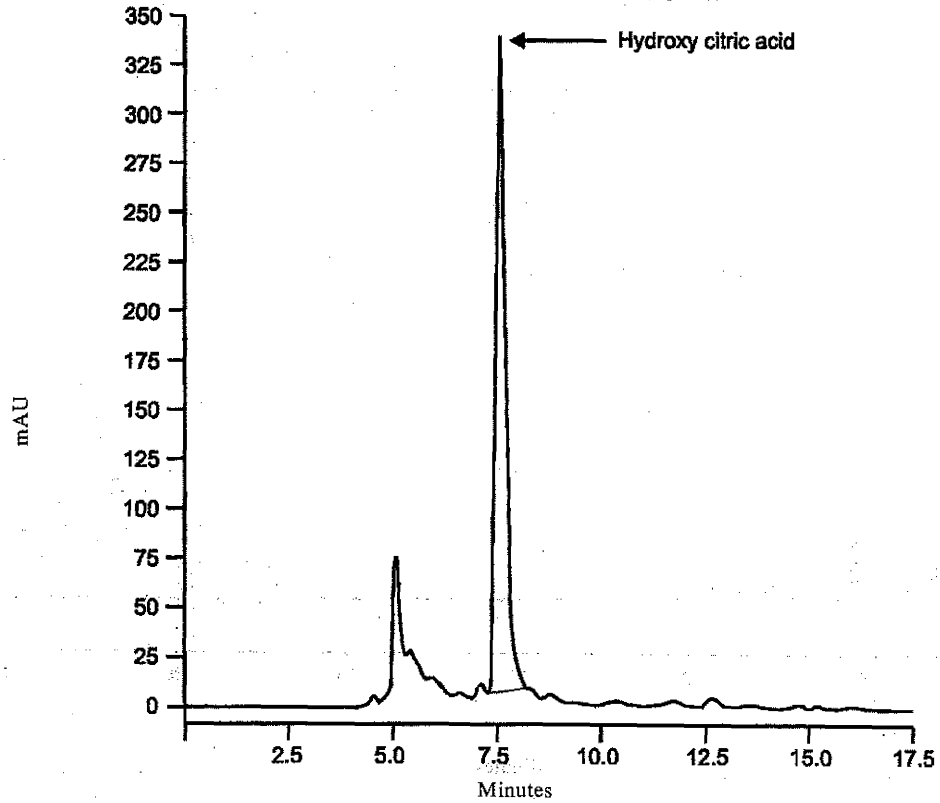


HPLC Chromatogram of Garcinia

Garcinia Aqueous Extract

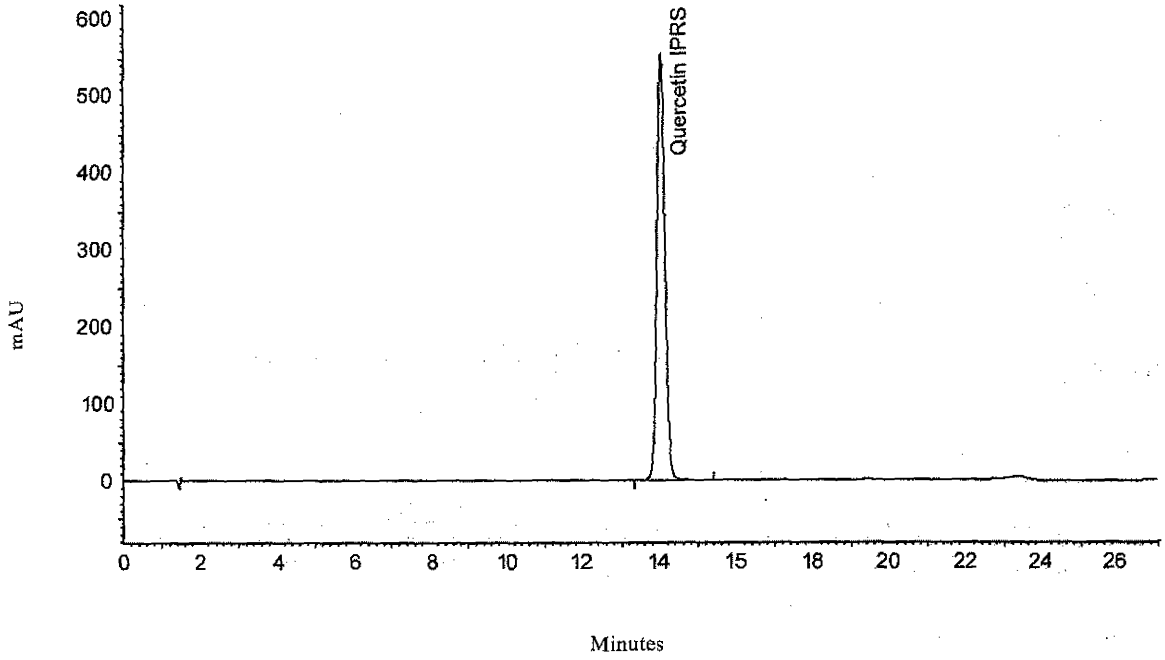


HPLC Chromatogram of hydroxy citric acid IPRS

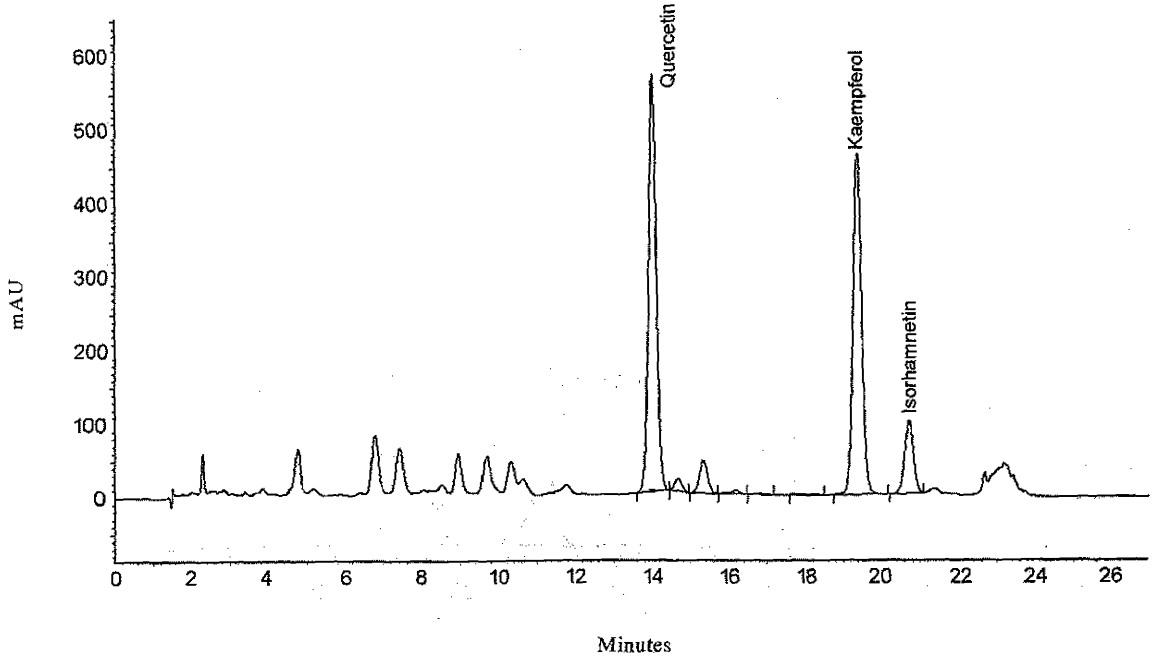


HPLC Chromatogram of Garcinia Aqueous Extract

Ginkgo Dry Extract

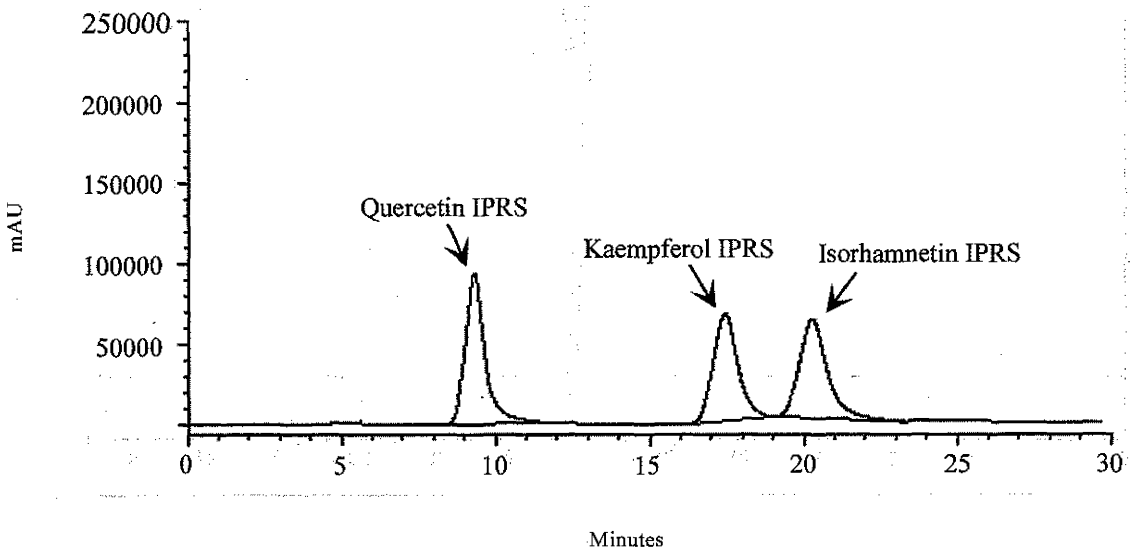


HPLC Chromatogram of quercetin IPRS

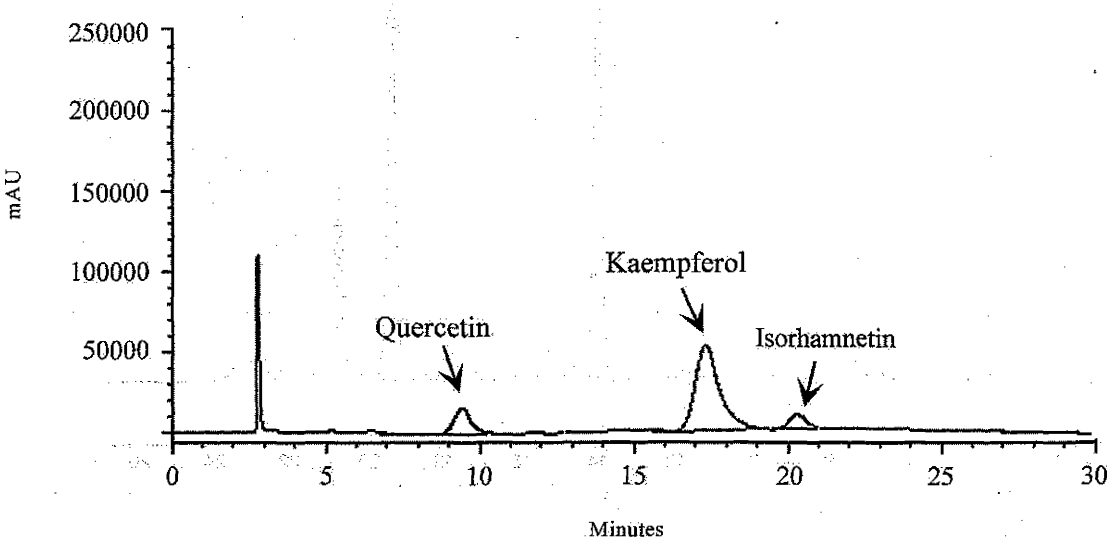


HPLC Chromatogram of Ginkgo Dry Extract

Ginkgo Tablets

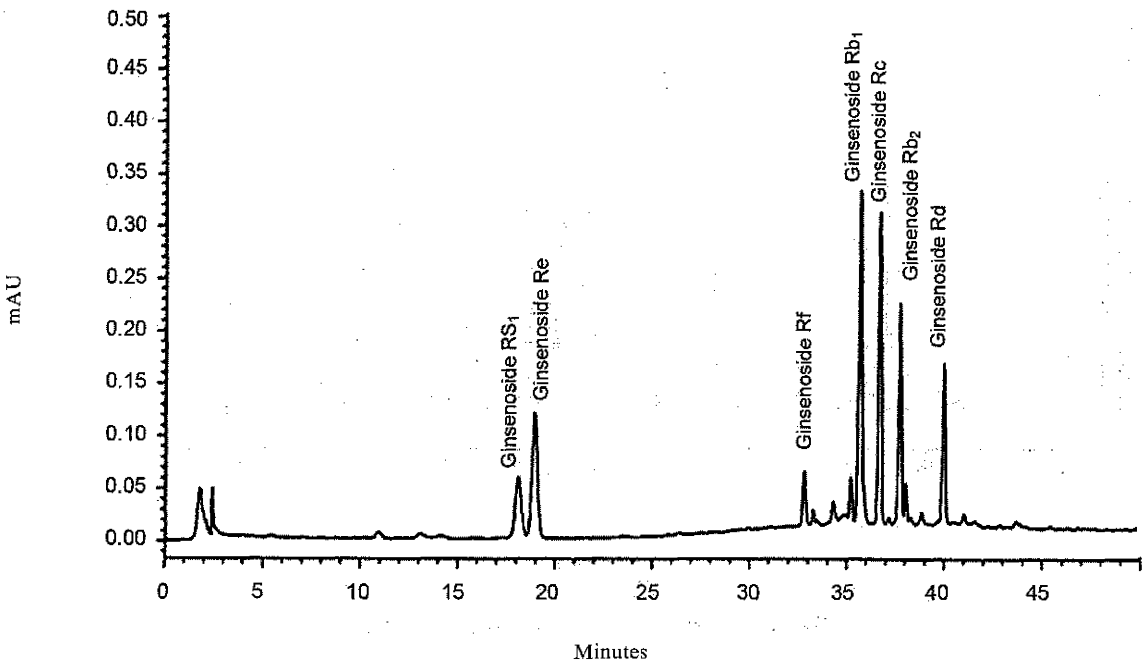


HPLC Chromatogram of quercetin IPRS, kaempferol IPRS and isorhamnetin IPRS



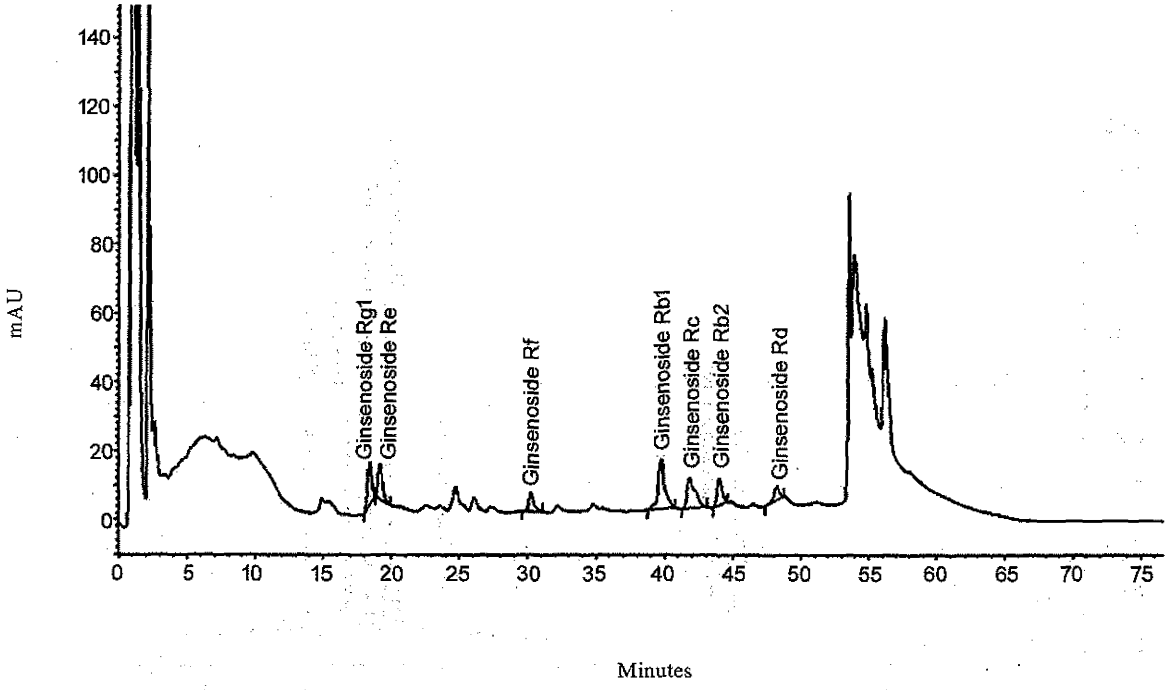
HPLC Chromatogram of Ginkgo Tablets

Ginseng

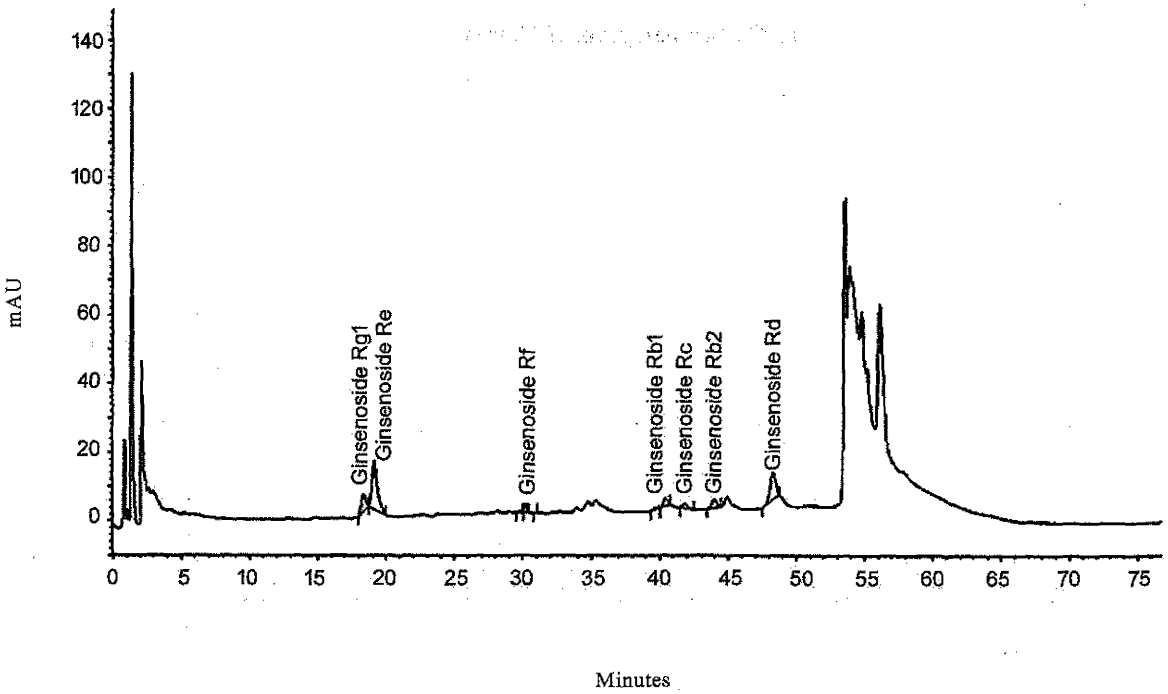


HPLC Chromatogram of Ginseng

Ginseng Dry Extract

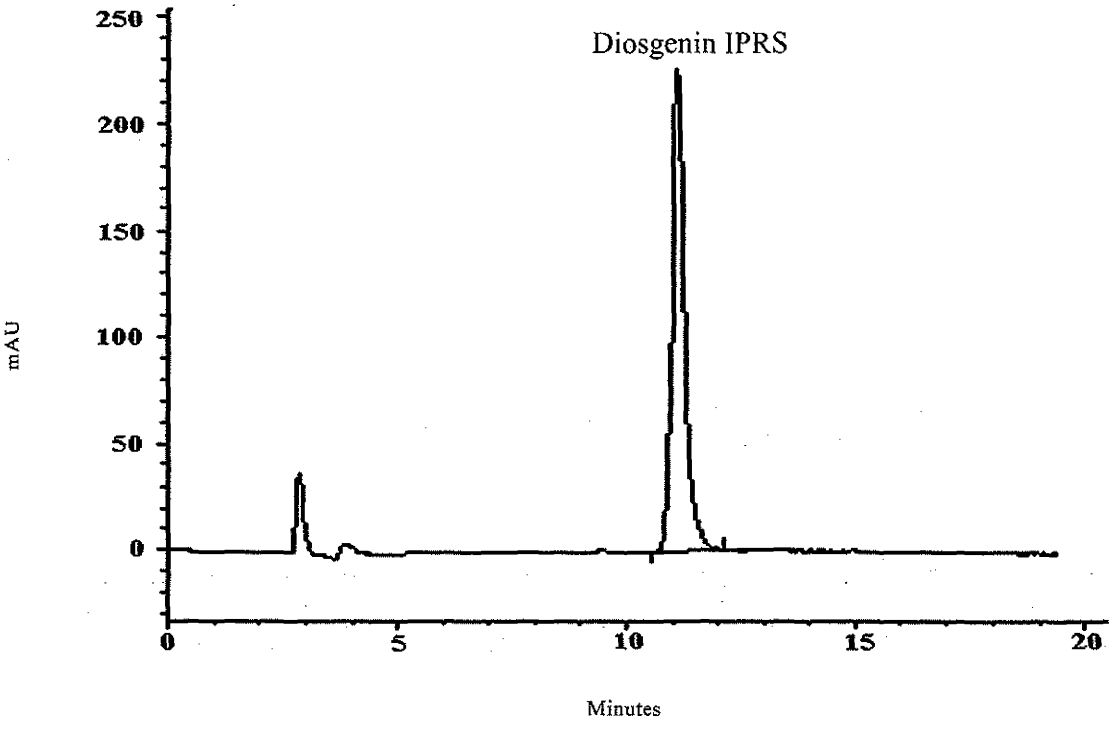


HPLC Chromatogram of ginseng dry extract

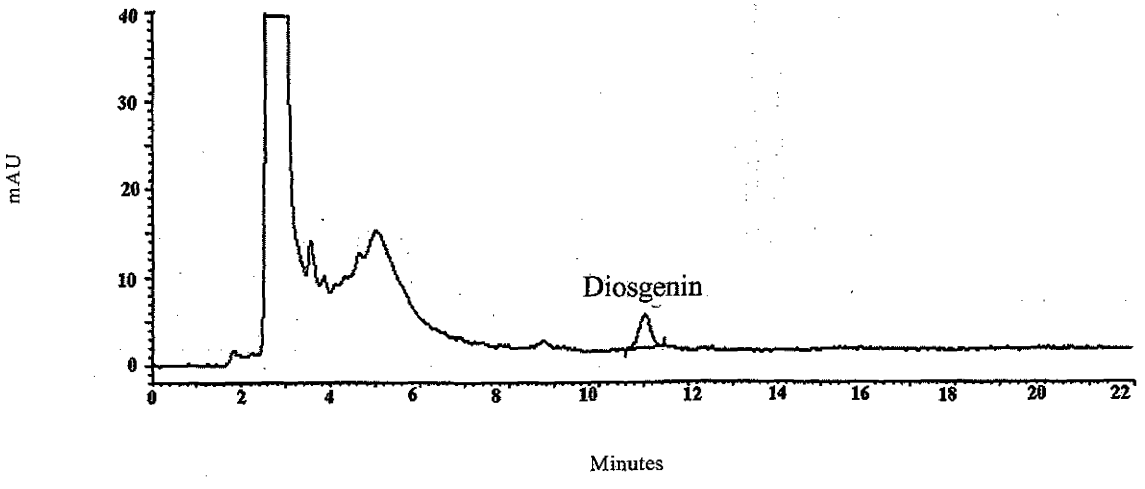


HPLC Chromatogram of Ginseng Dry Extract

Gokhru

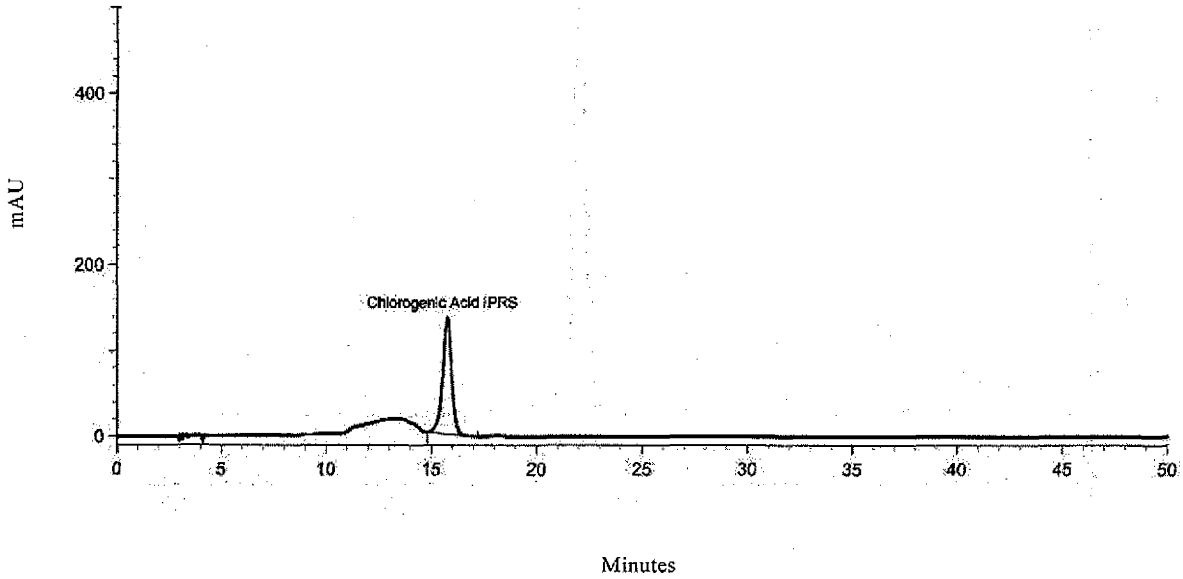


HPLC Chromatogram of diosgenin IPRS

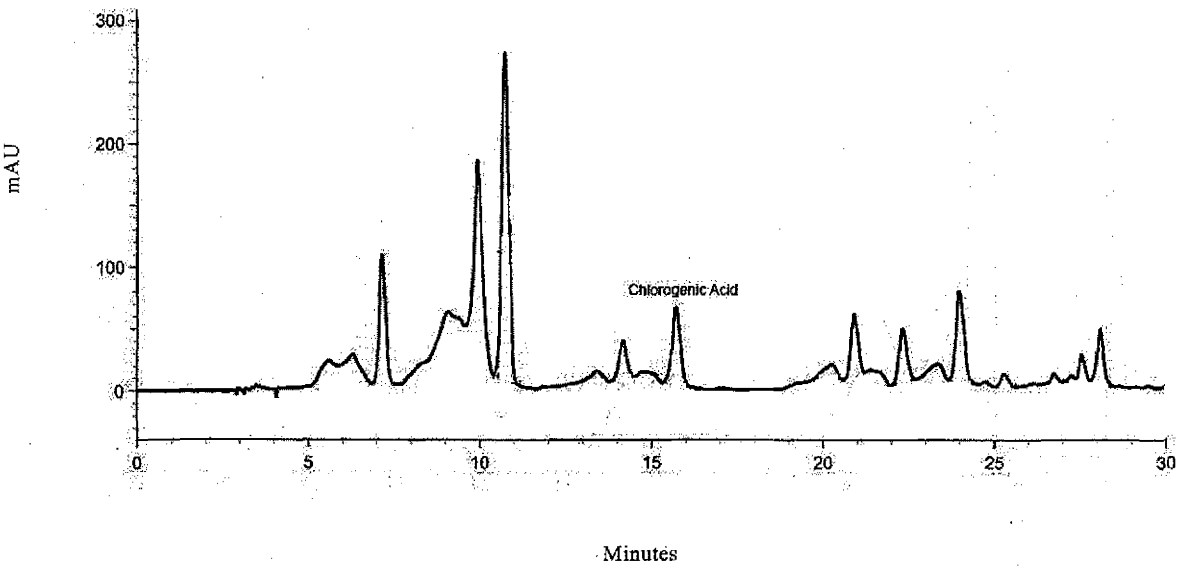


HPLC Chromatogram of Gokhru

Green Coffee Bean Extract

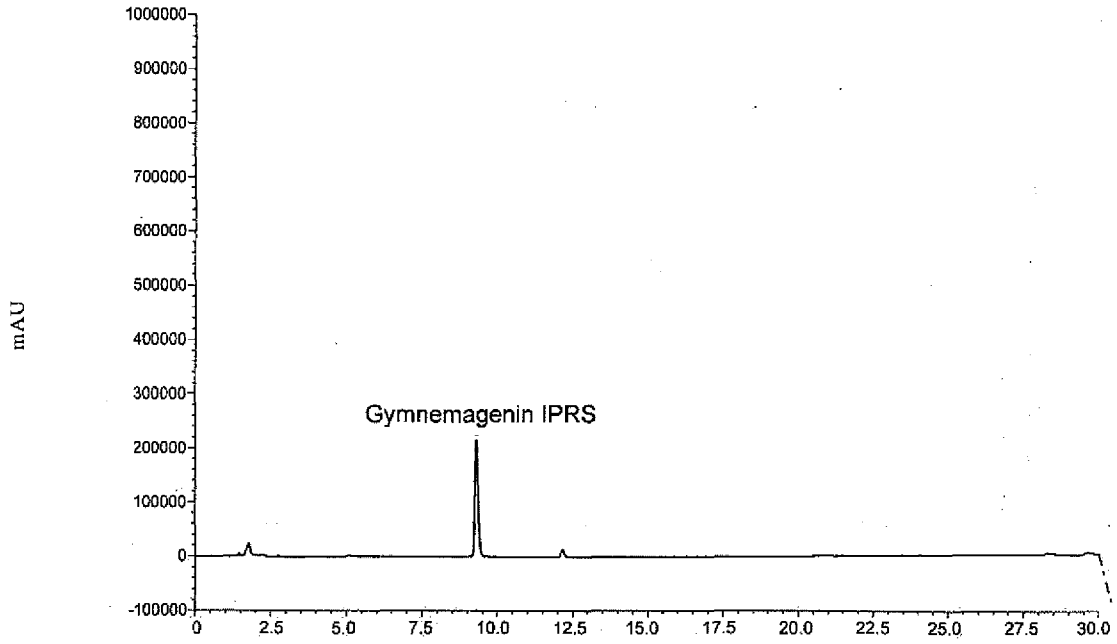


HPLC Chromatogram of chlorogenic acid IPRS

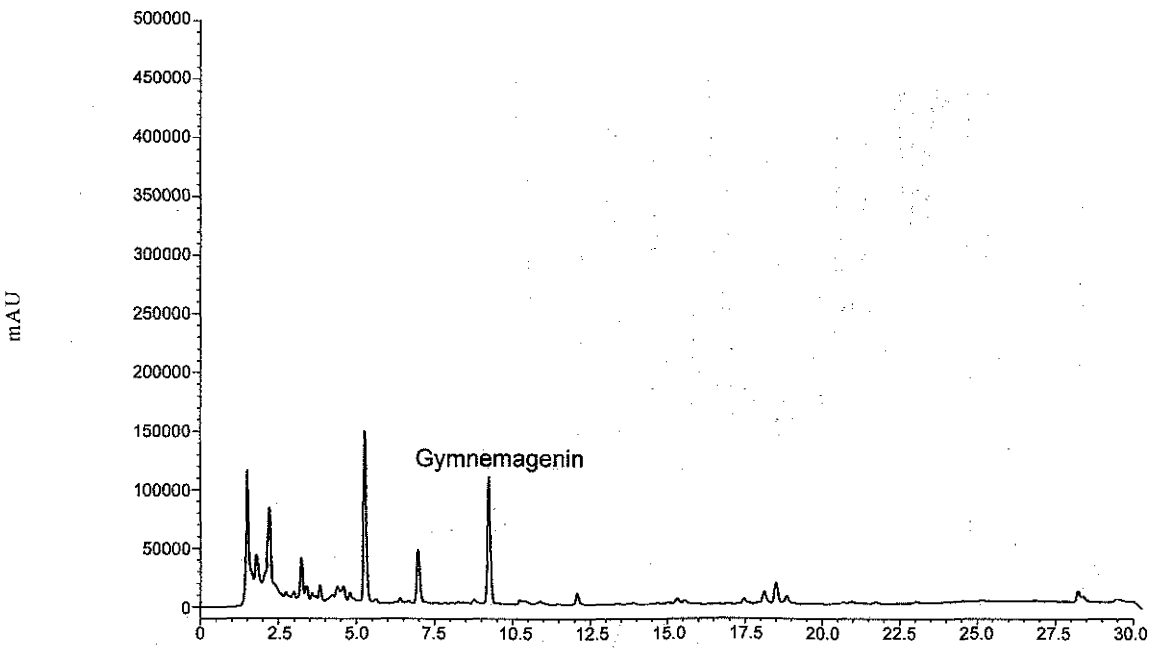


HPLC Chromatogram of Green Coffee Bean Extract

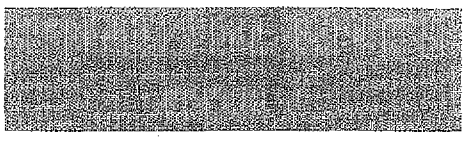
Gudmar Dry Extract



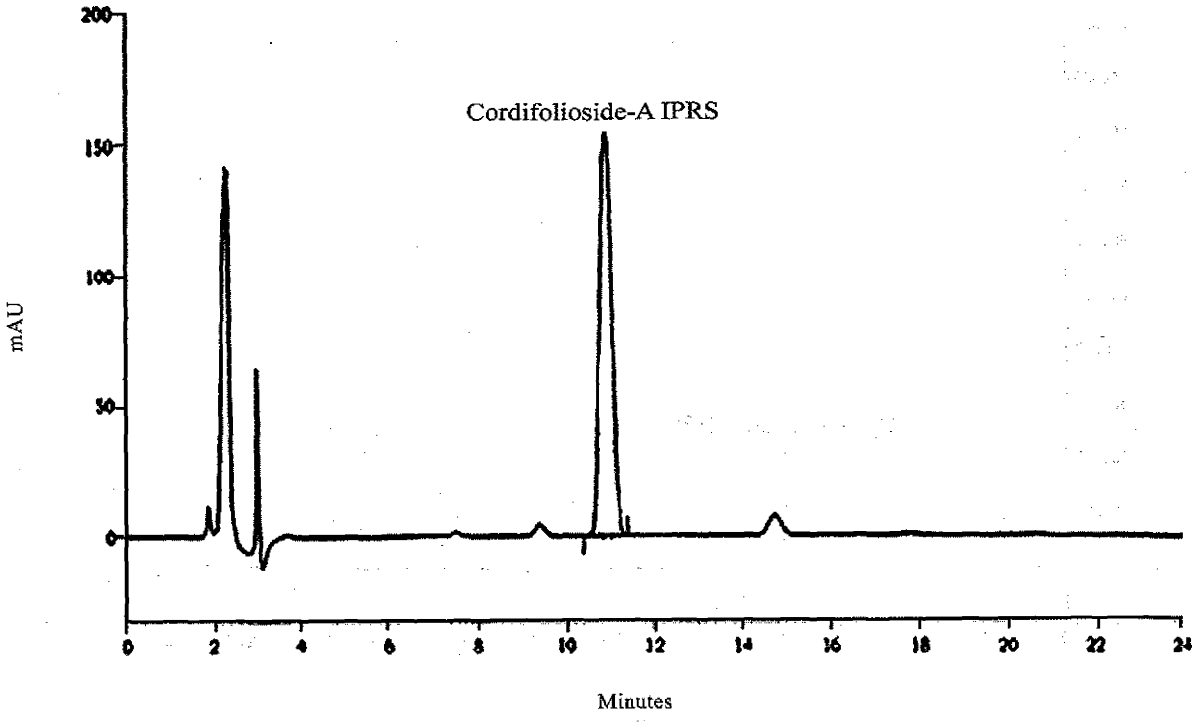
HPLC Chromatogram of gymnemagenin IPRS



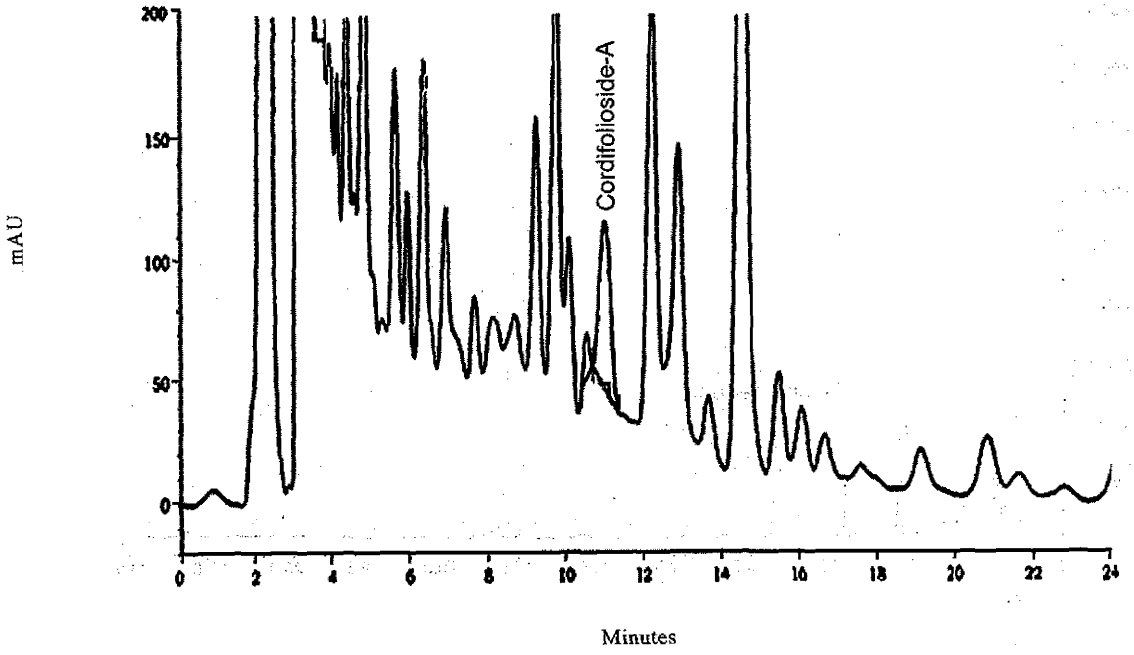
HPLC Chromatogram of Gudmar Dry Extract



Guduchi

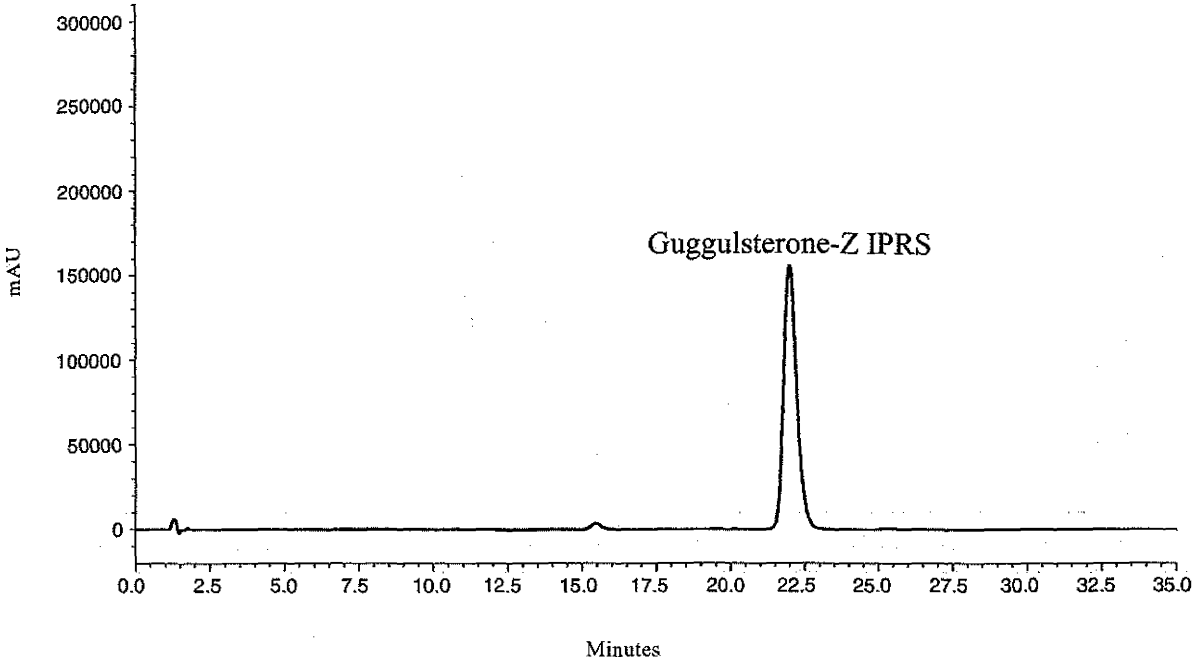


HPLC Chromatogram of cordifolioside-A IPRS

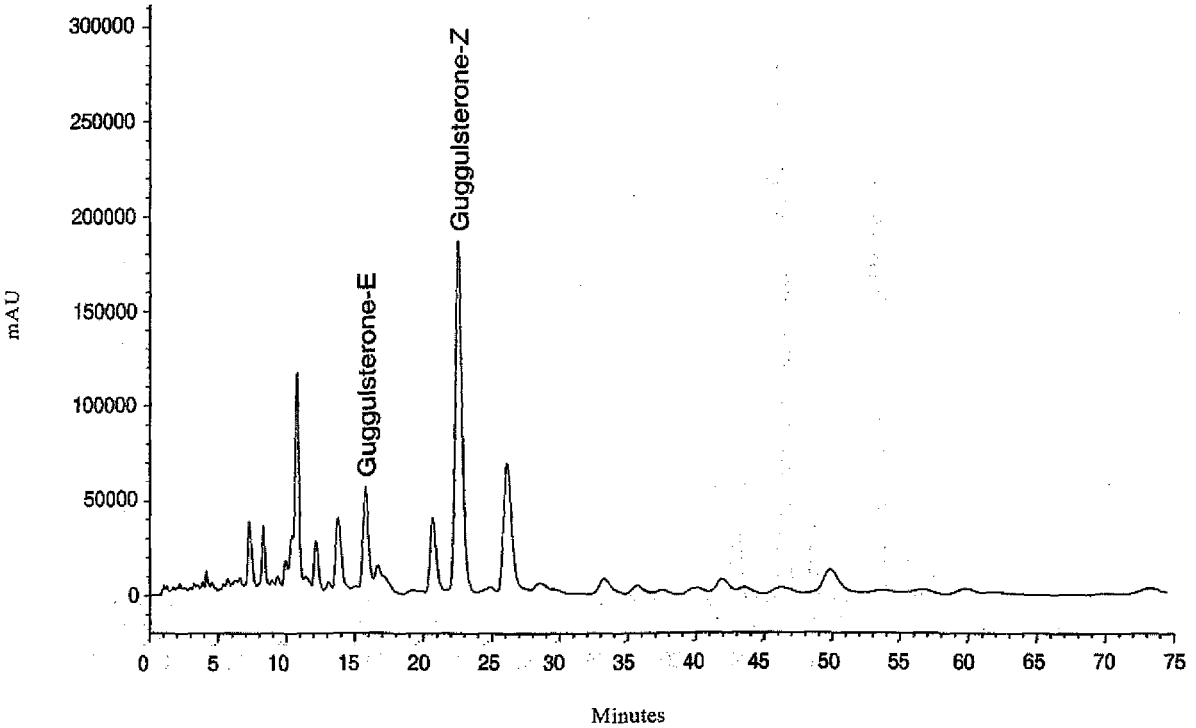


HPLC Chromatogram of Guduchi

Guggul Resin

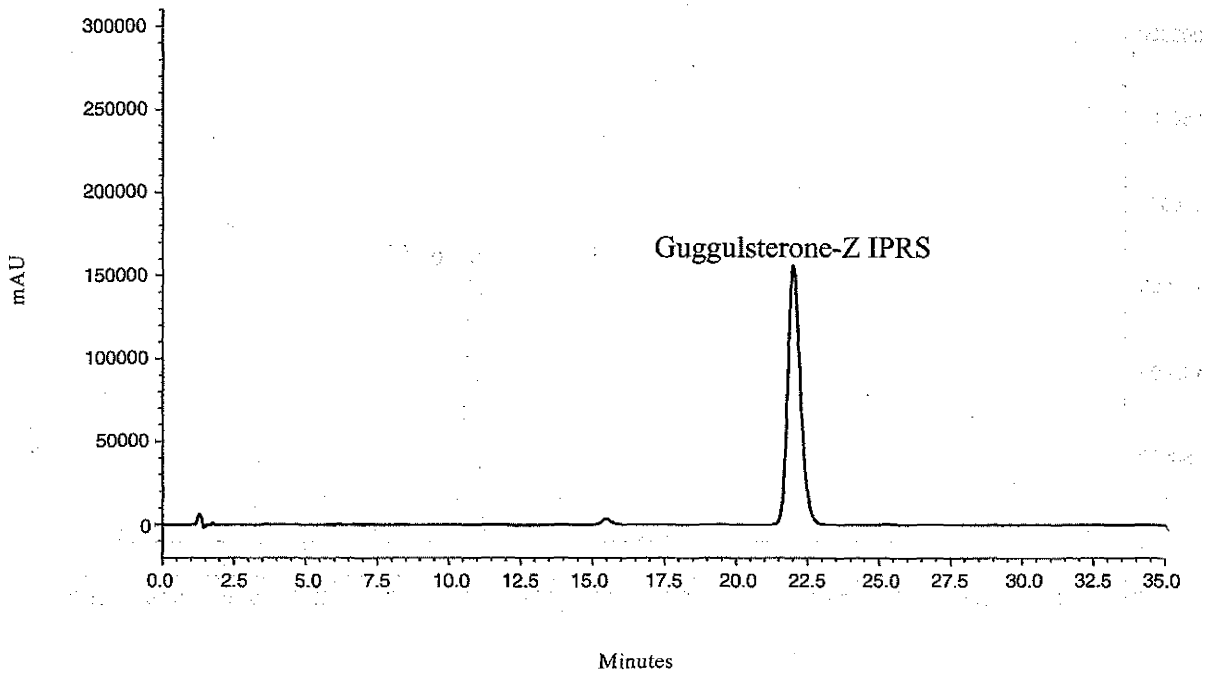


HPLC Chromatogram of guggulsterone (Z) IPRS

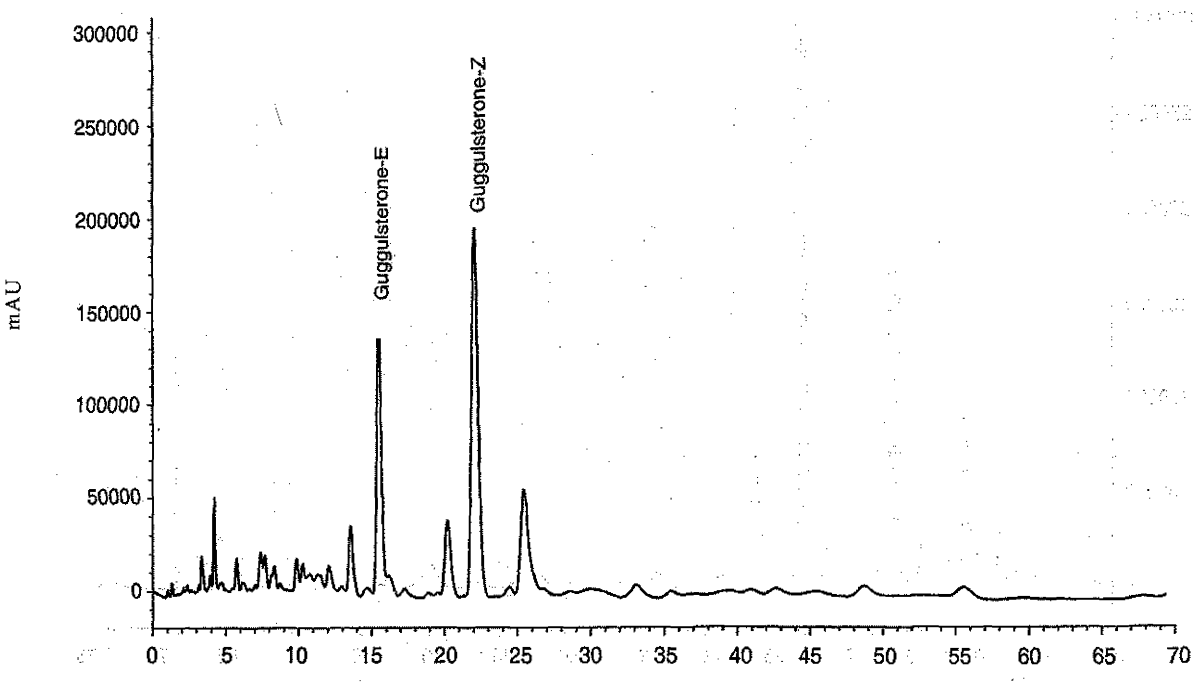


HPLC Chromatogram of Guggul Resin

Guggulipid

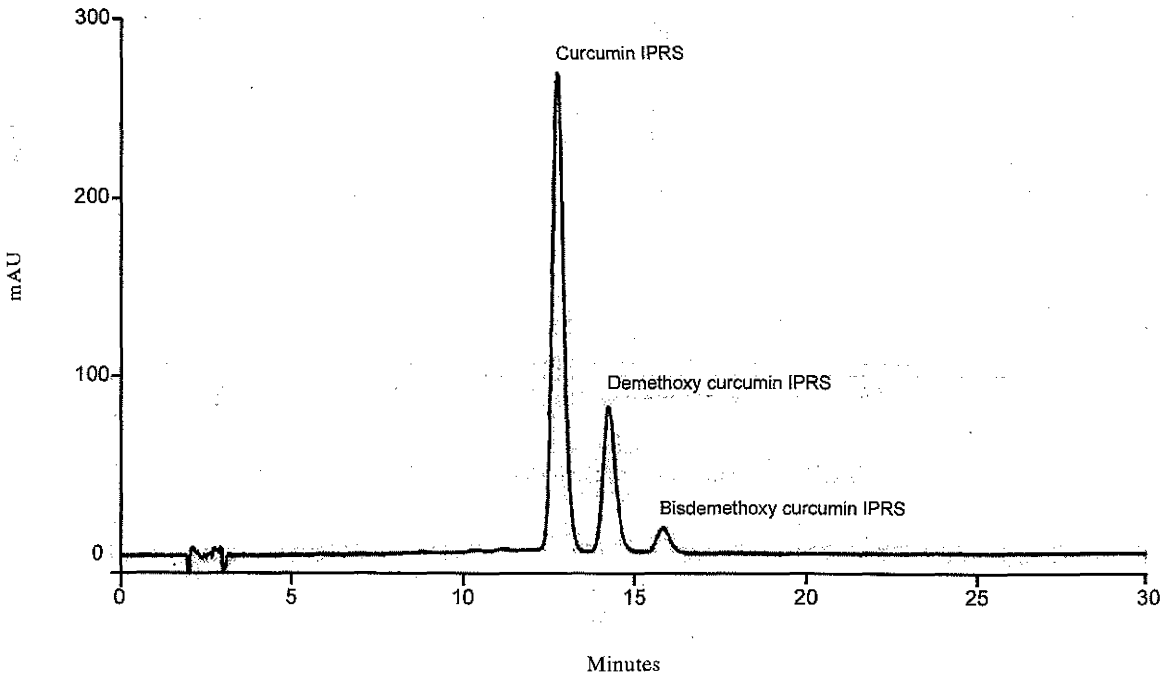


HPLC Chromatogram of guggulsterone-Z IPRS

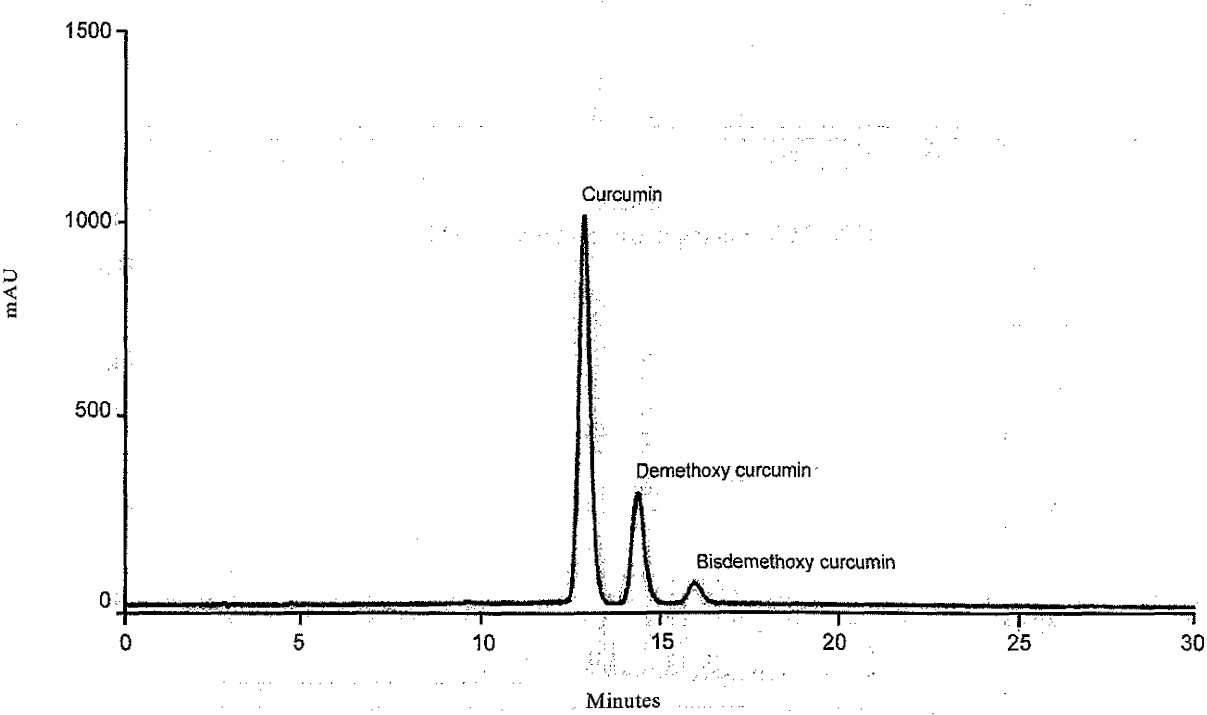


HPLC Chromatogram of Gugulipid

Haridra Dry Extract

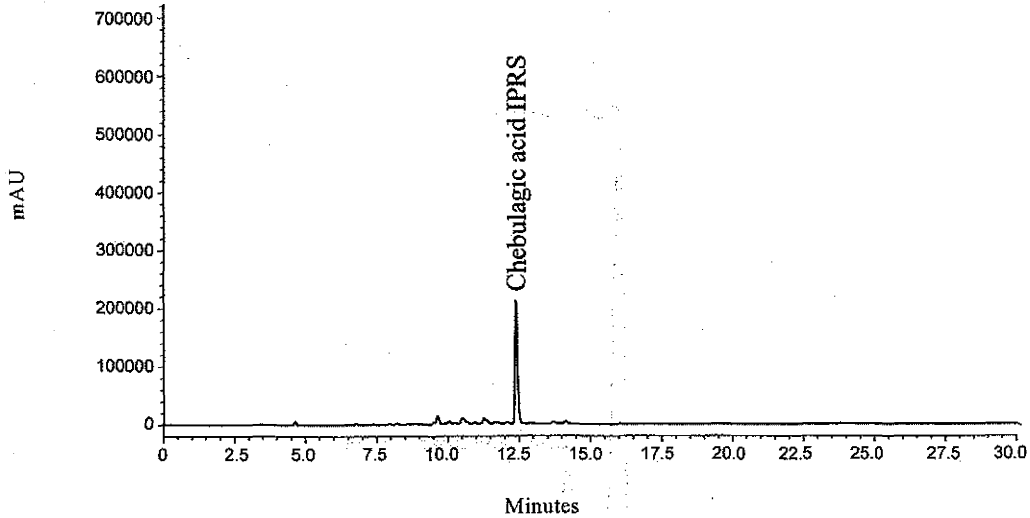


HPLC Chromatogram of curcumin IPRS, demethoxycurcumin IPRS and bis-demethoxycurcumin IPRS

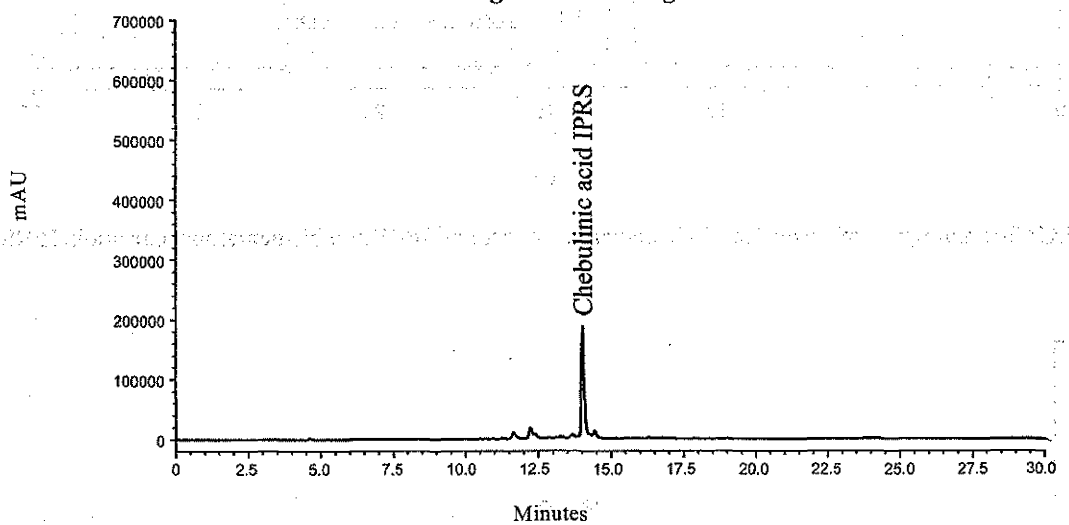


HPLC Chromatogram of Haridra Dry Extract

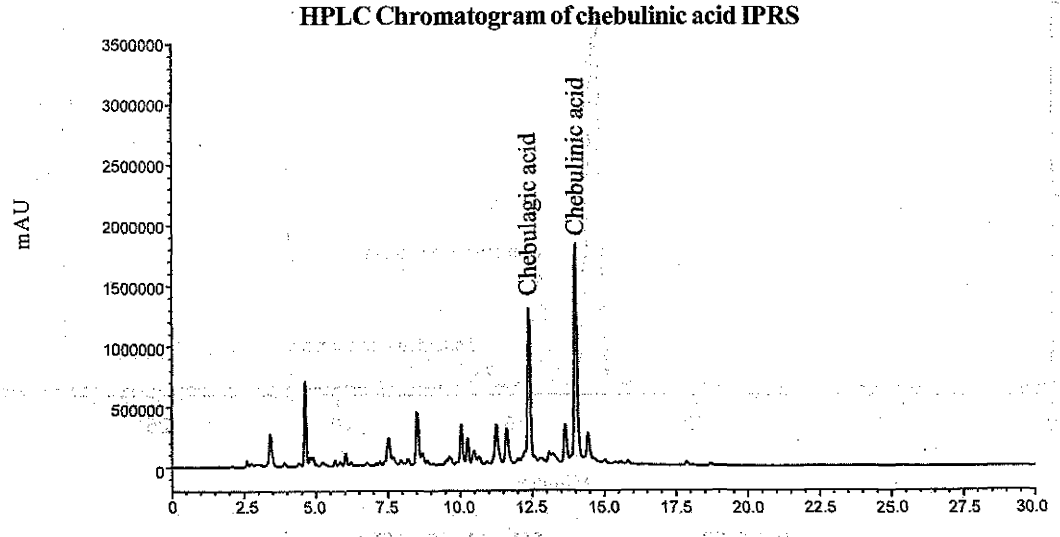
Haritaki



HPLC Chromatogram of chebulagic acid IPRS

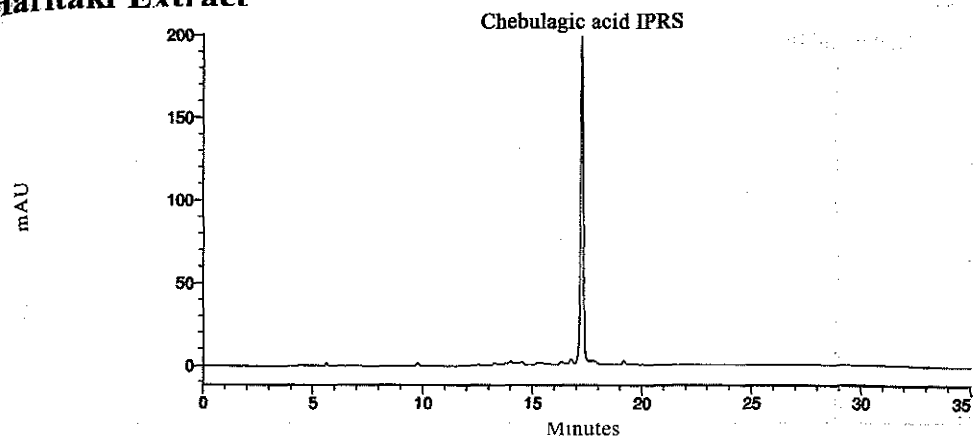


HPLC Chromatogram of chebulinic acid IPRS

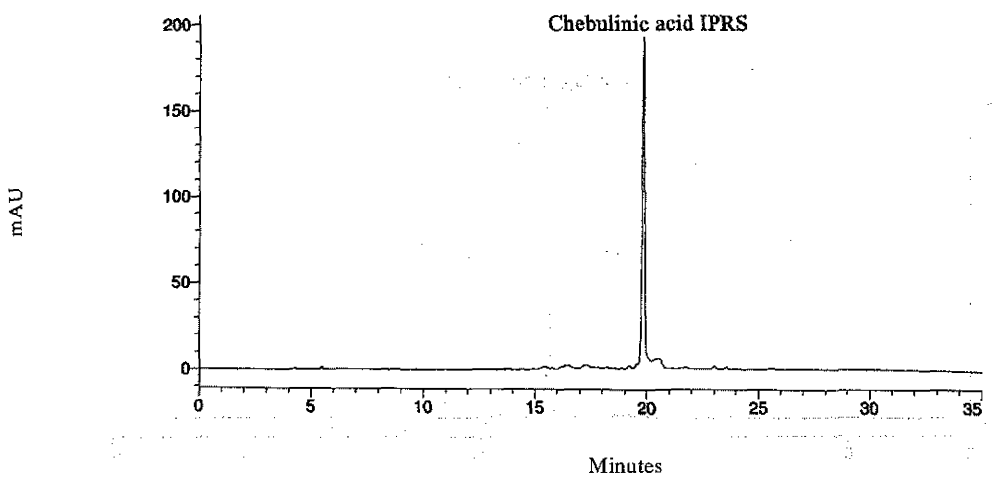


HPLC Chromatogram of Haritaki

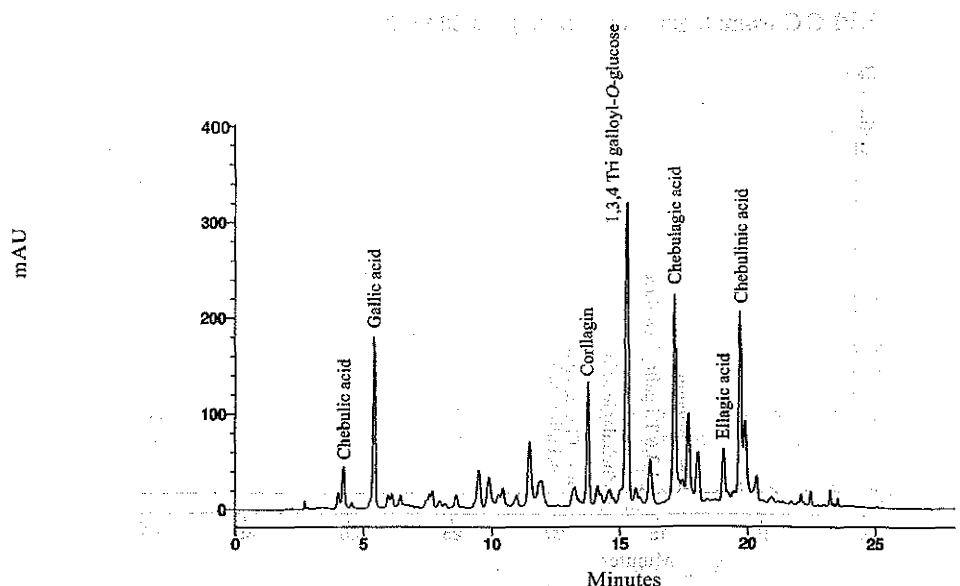
Haritaki Extract



HPLC Chromatogram of chebulagic acid IPRS

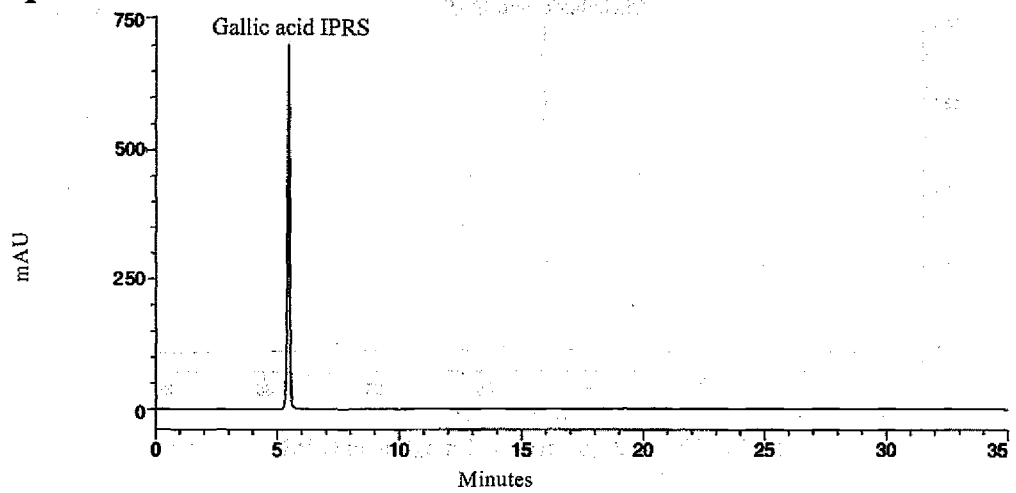


HPLC Chromatogram of chebulinic acid IPRS

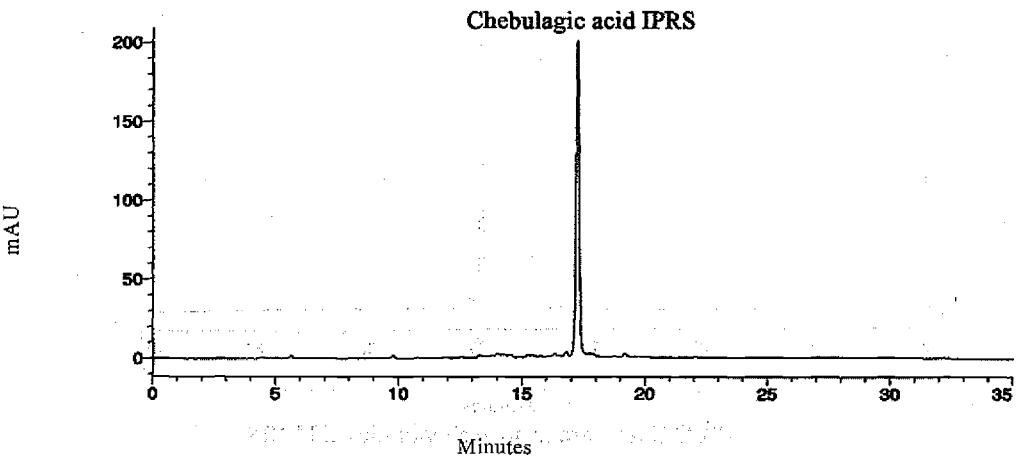


HPLC Chromatogram of Haritaki Extract

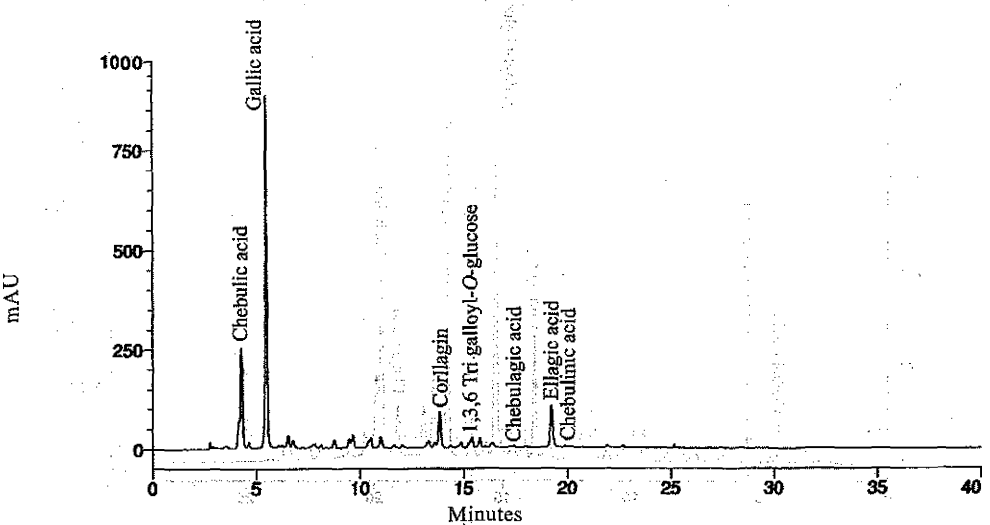
Haritaki Aqueous Extract



HPLC Chromatogram of gallic acid IPRS

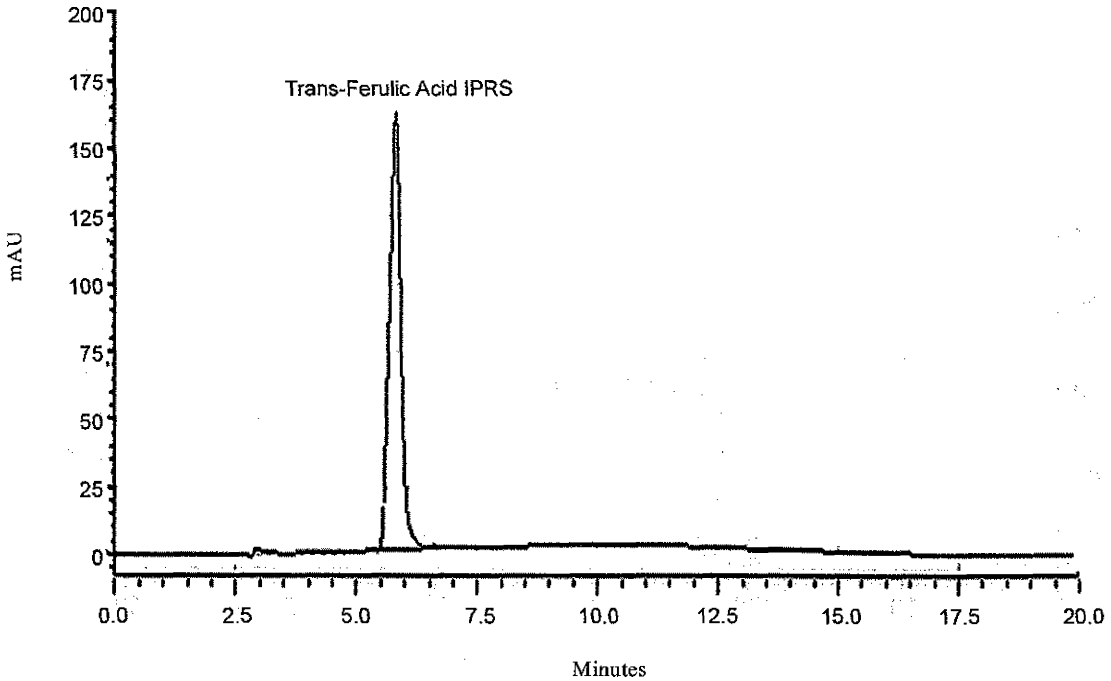


HPLC Chromatogram of chebulagic acid IPRS

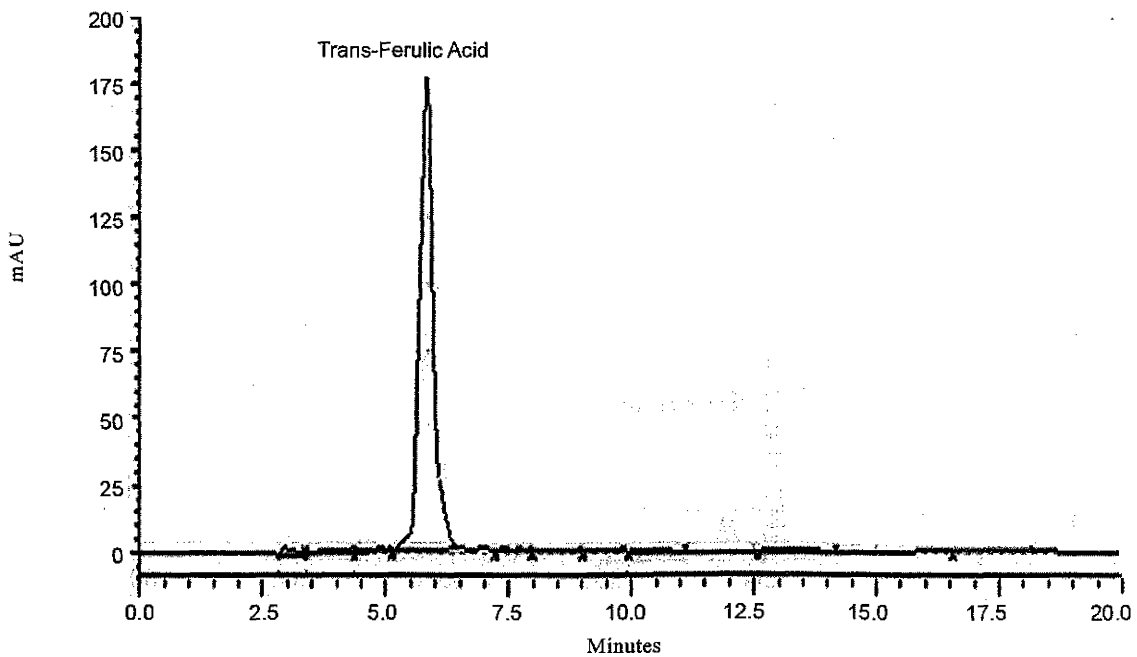


HPLC Chromatogram of Haritaki Aqueous Extract

Hingu

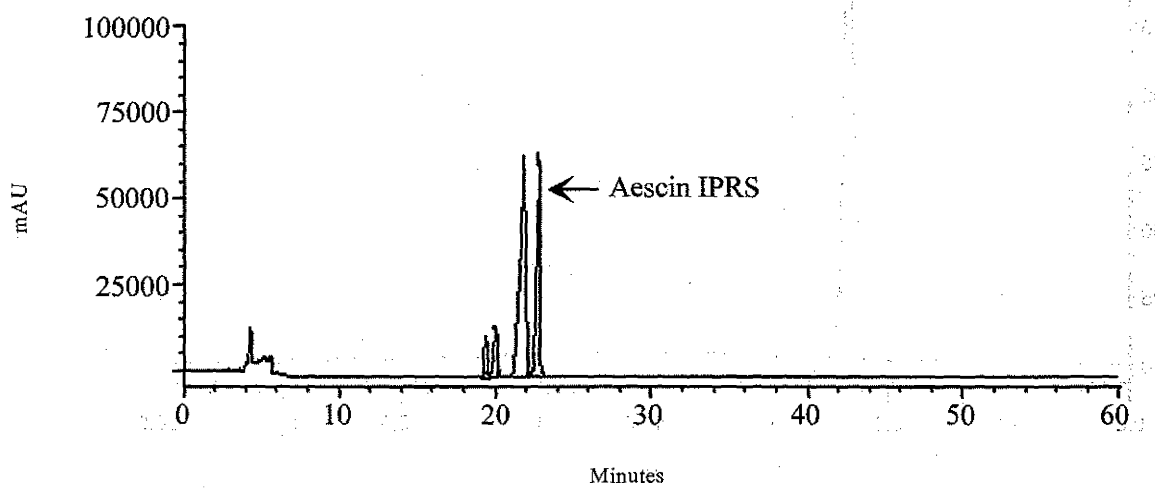


HPLC Chromatogram of trans-ferulic acid IPRS

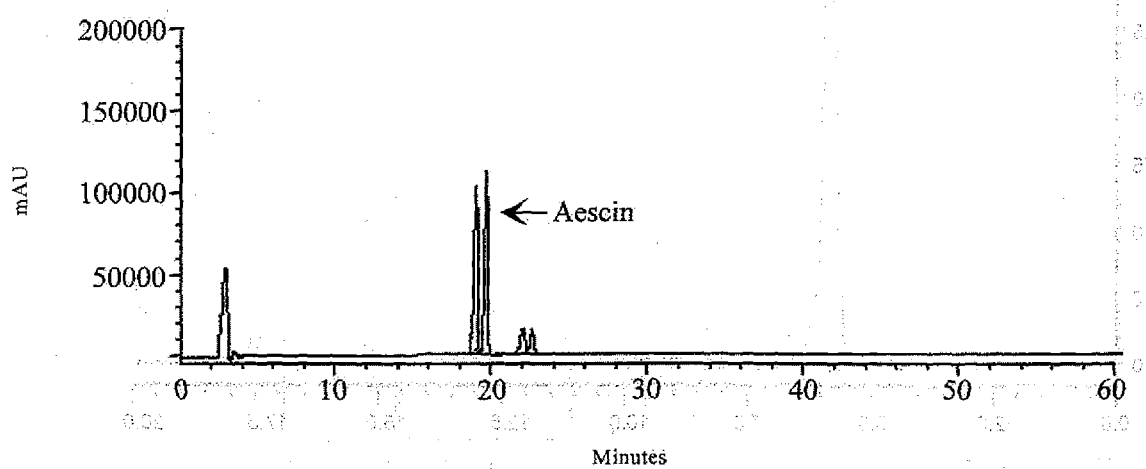


HPLC Chromatogram of Hingu

Horse Chestnut Dry Extract

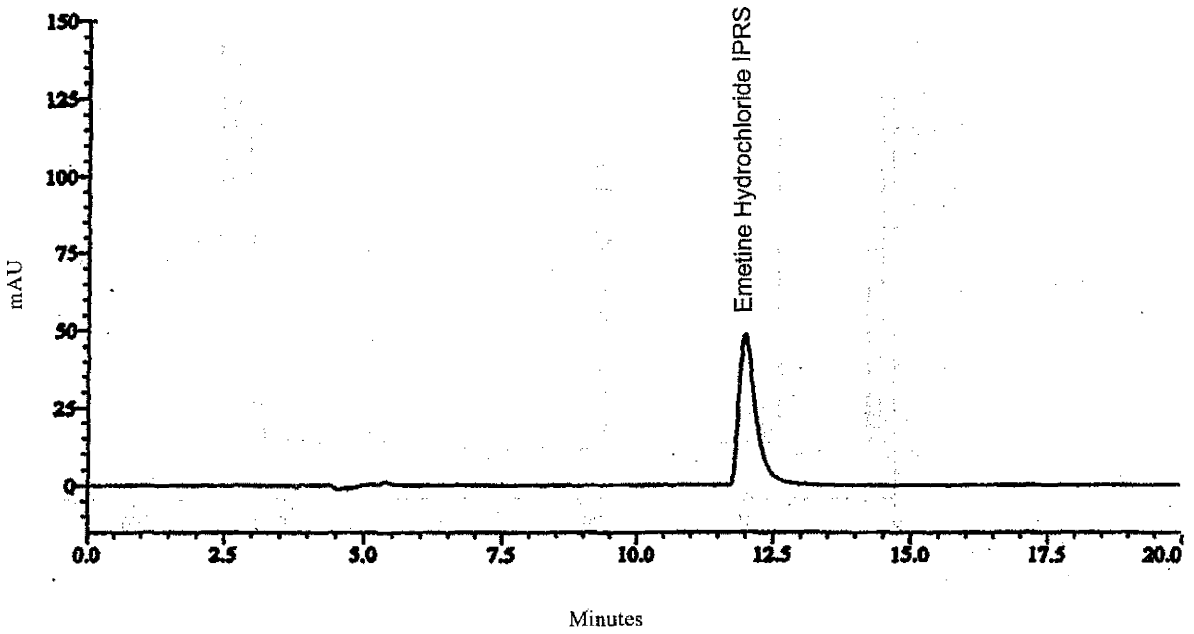


HPLC Chromatogram of aescin IPRS

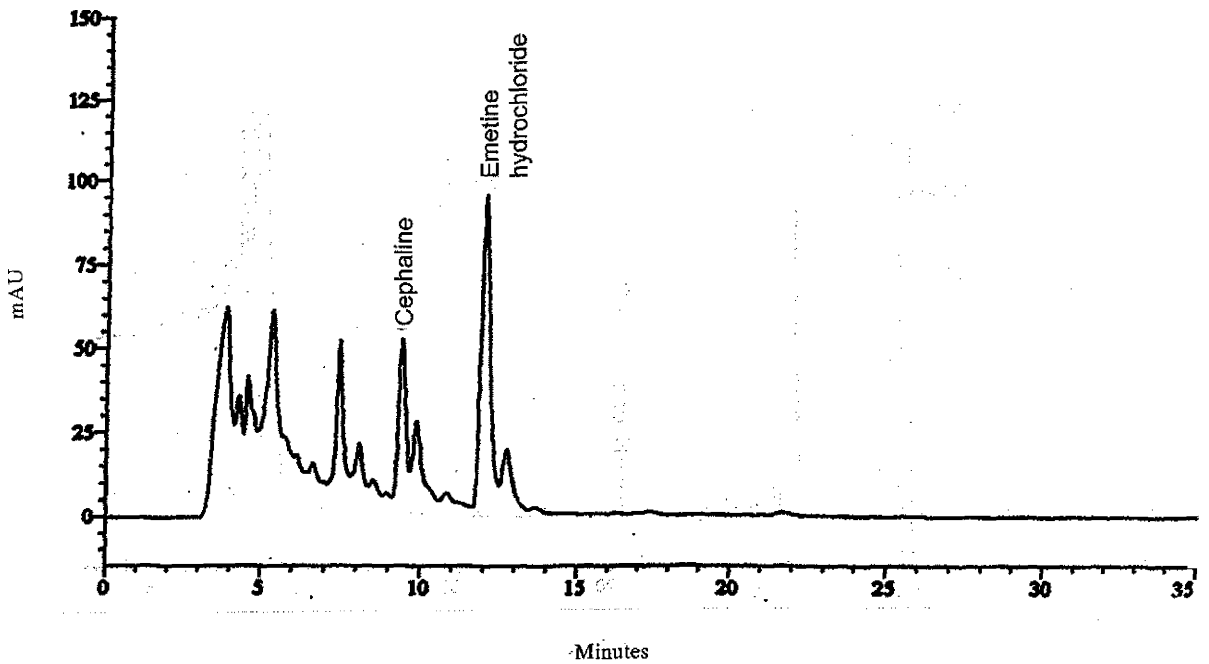


HPLC Chromatogram of Horse Chestnut Dry Extract

Ipecac Tincture

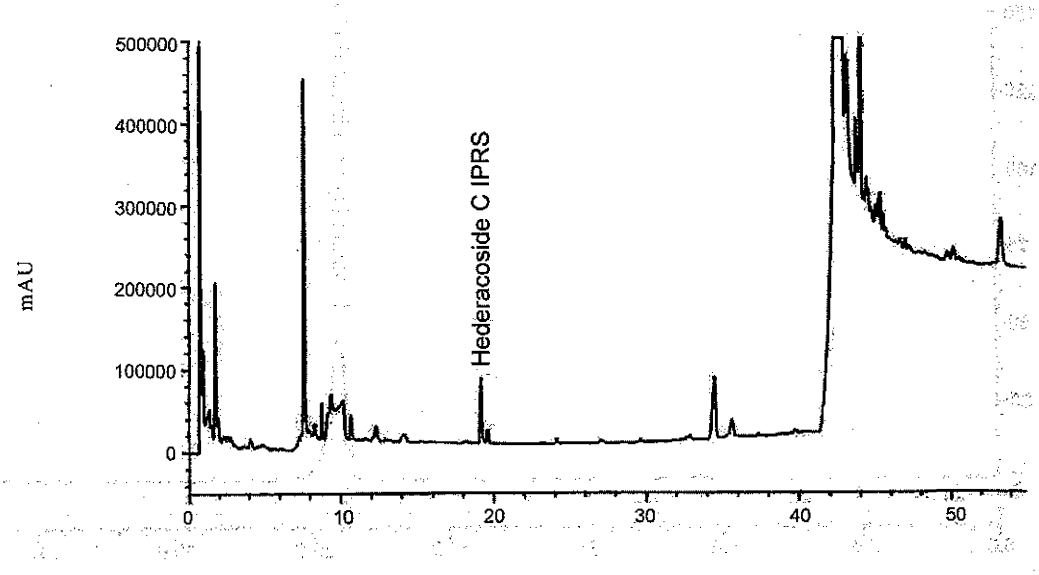


HPLC Chromatogram of emetine hydrochloride IPRS

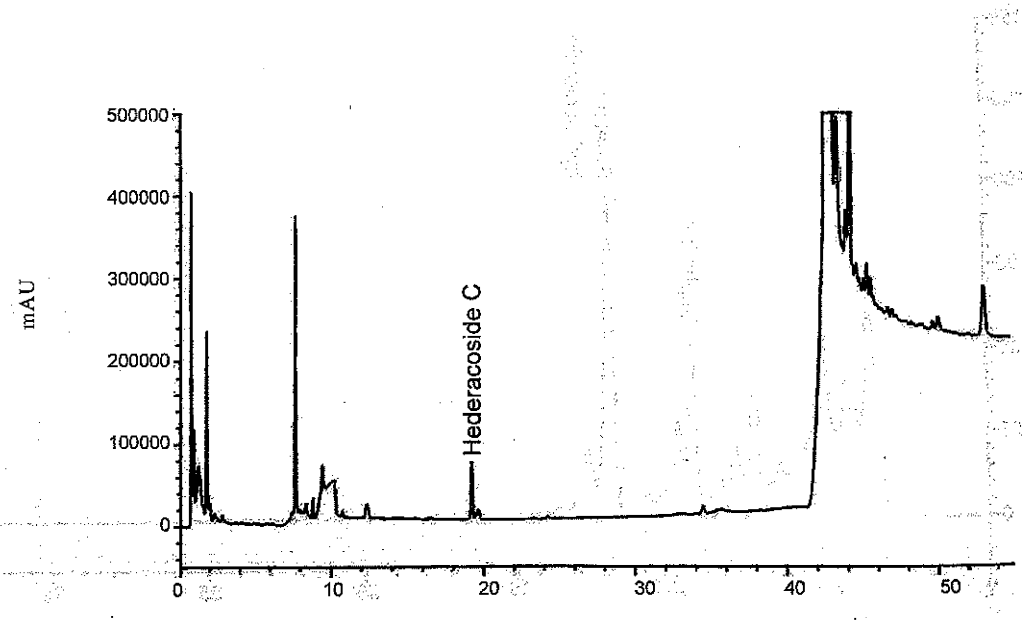


HPLC Chromatogram of Ipecac Tincture

Ivy Leaf

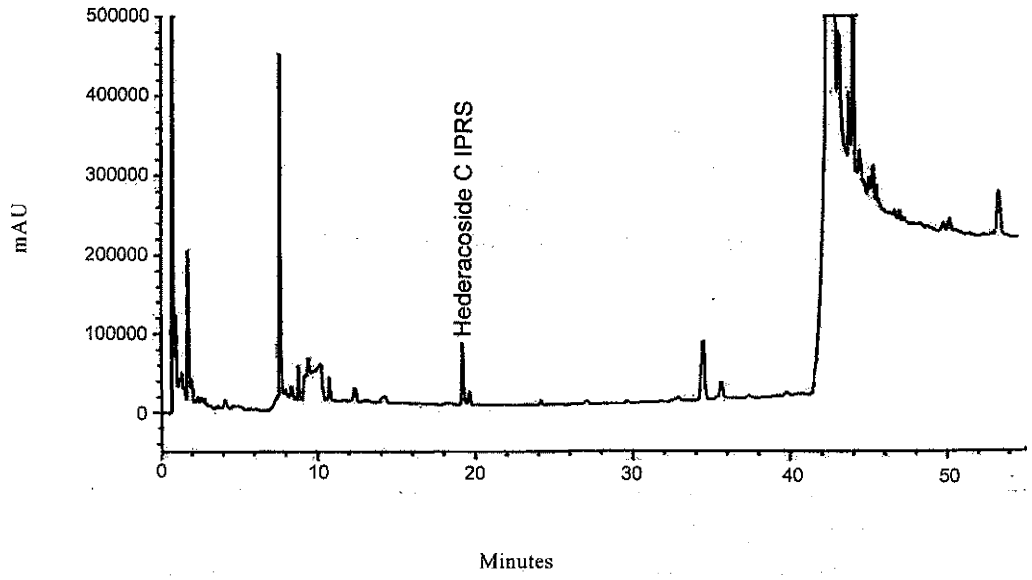


HPLC Chromatogram of hederacoside C IPRS

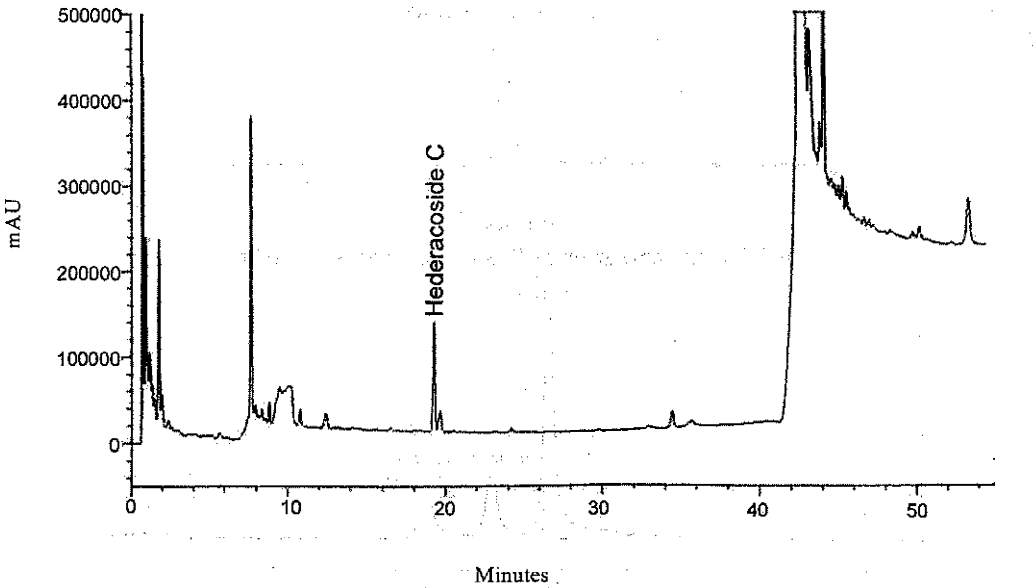


HPLC Chromatogram of Ivy Leaf

Ivy Leaf Dry Extract

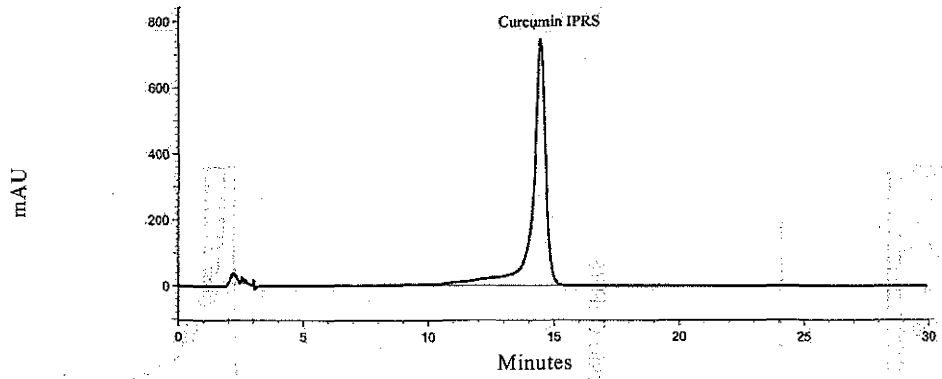


HPLC Chromatogram of hederacoside C IPRS

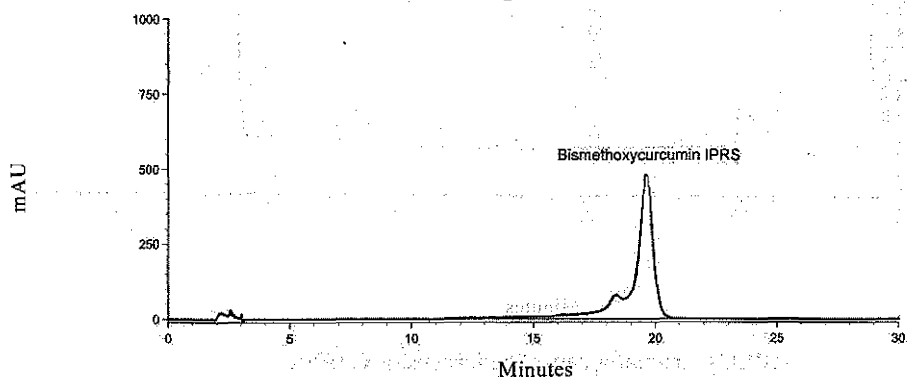


HPLC Chromatogram of Ivy Leaf Dry Extract

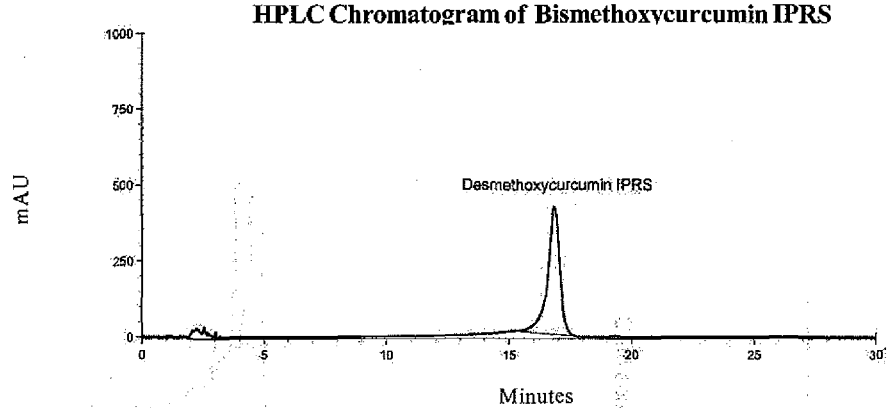
Jangali Haldi



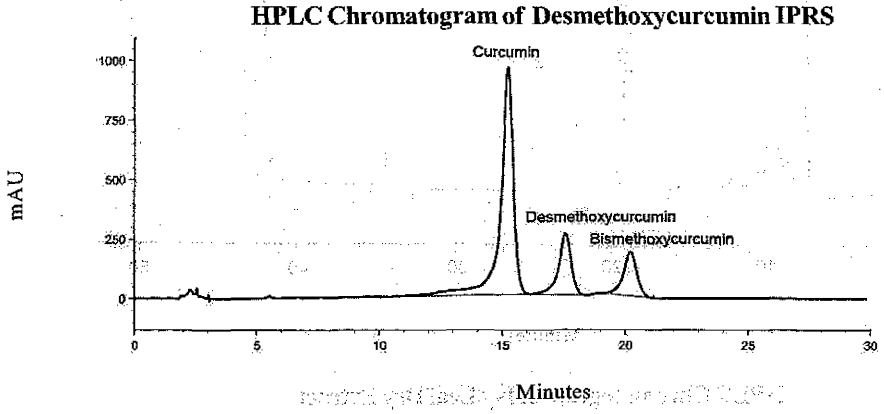
HPLC Chromatogram of Curcumin IPRS



HPLC Chromatogram of Bismethoxycurcumin IPRS

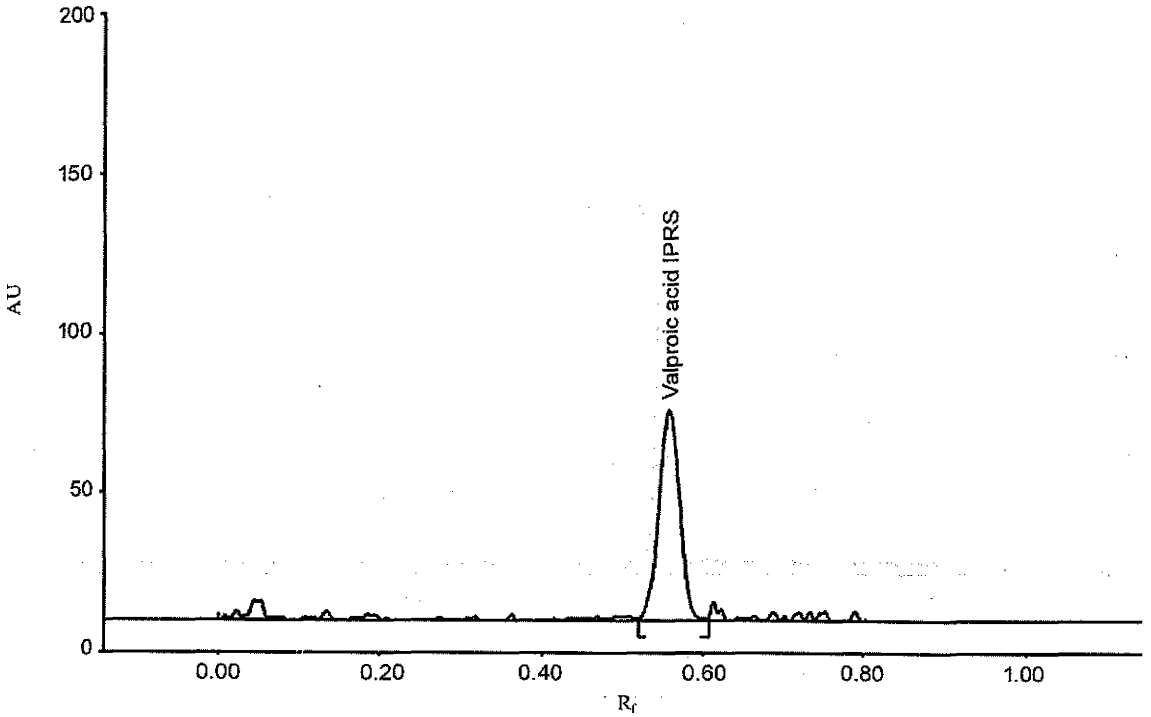


HPLC Chromatogram of Desmethoxycurcumin IPRS

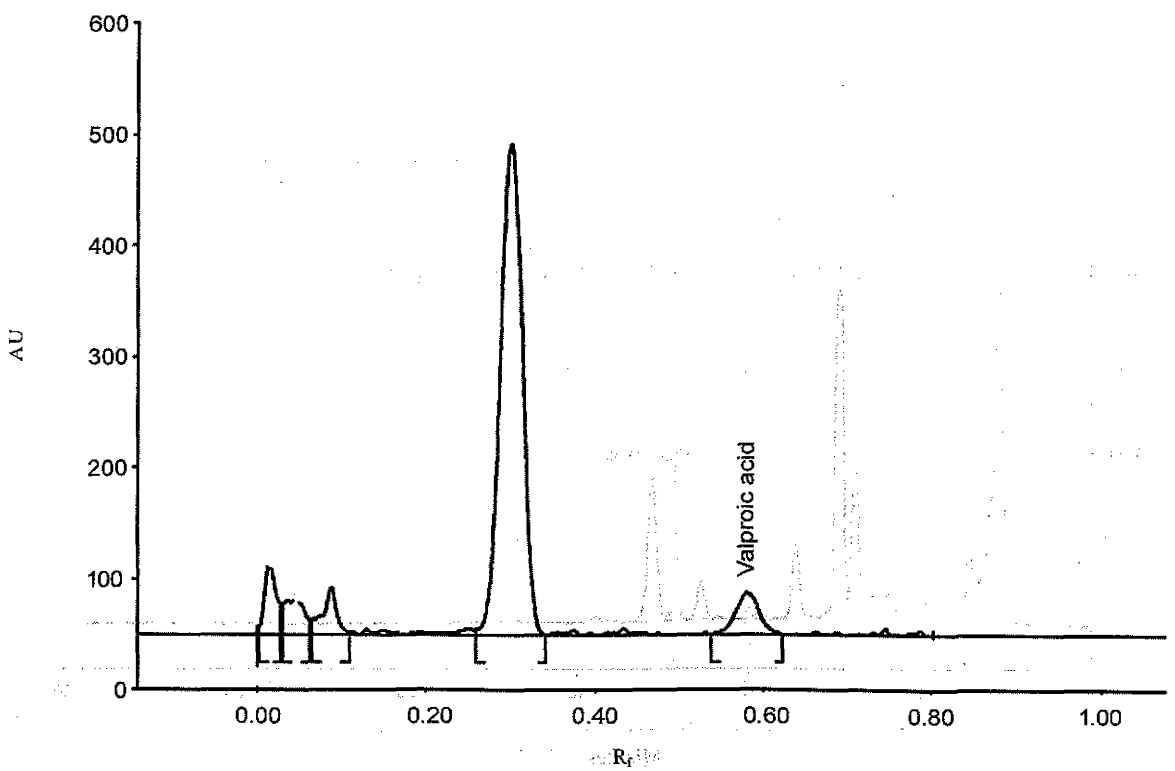


HPLC Chromatogram of Jangali Haldi

Jatamansi

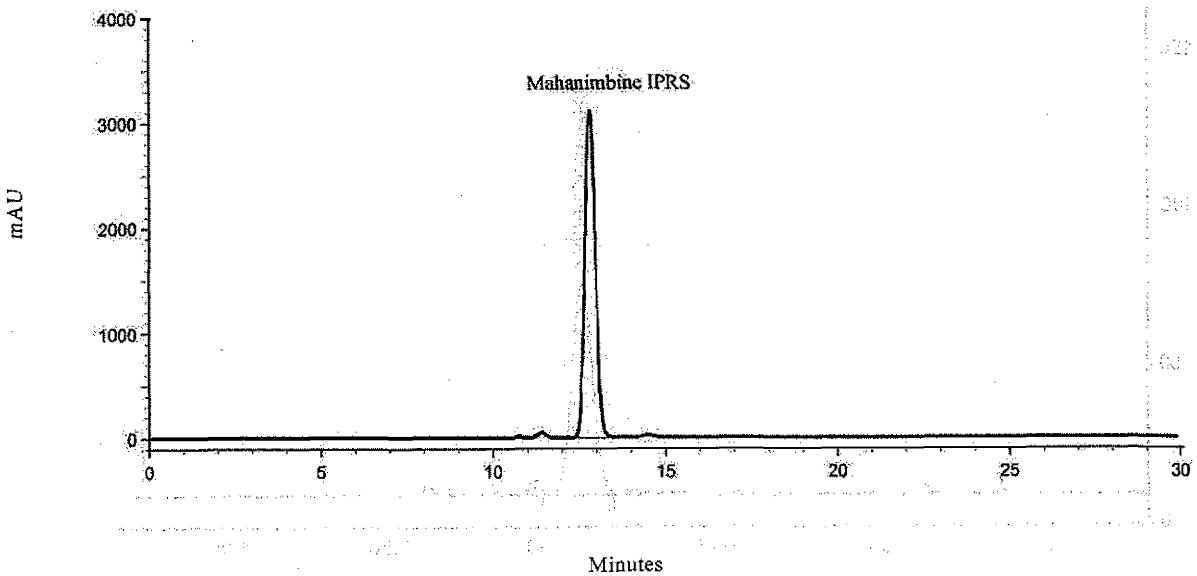


HPTLC Chromatogram of valproic acid IPRS

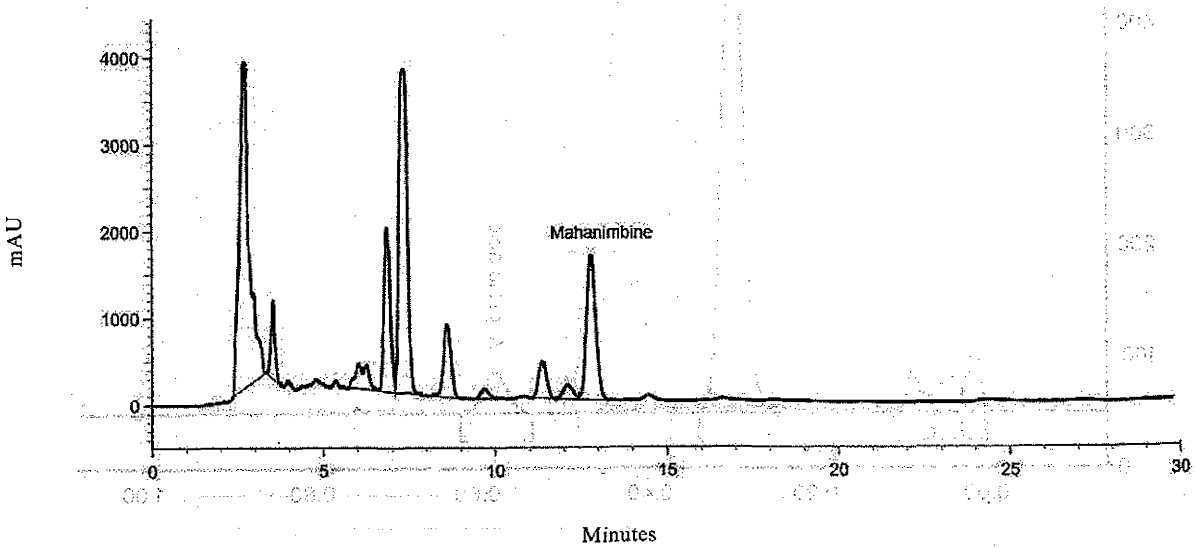


HPTLC Chromatogram of Jatamansi

Karipatta

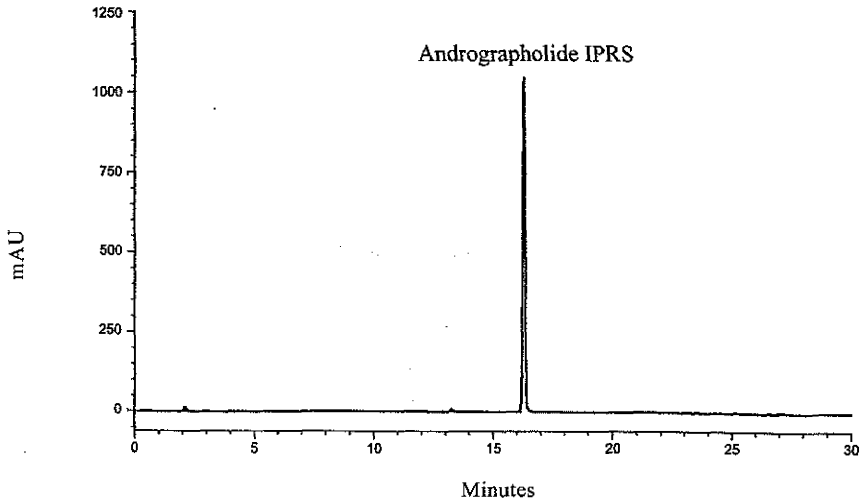


HPLC Chromatogram of Mahanimbine IPRS

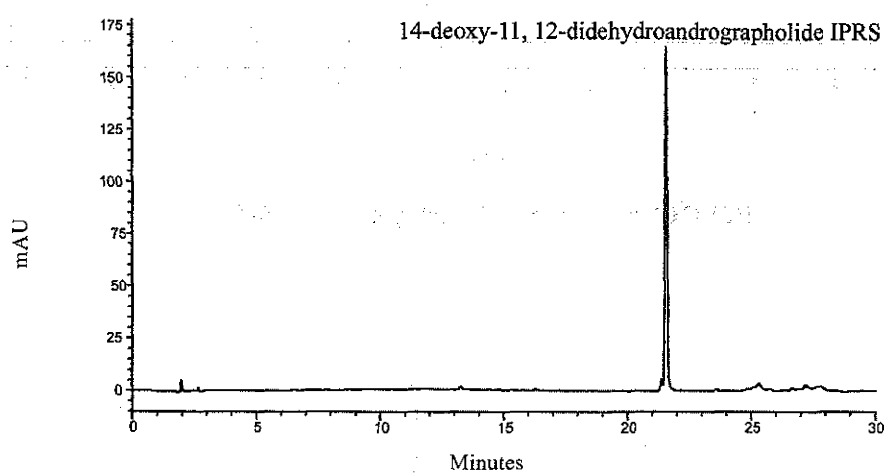


HPLC Chromatogram of Karipatta

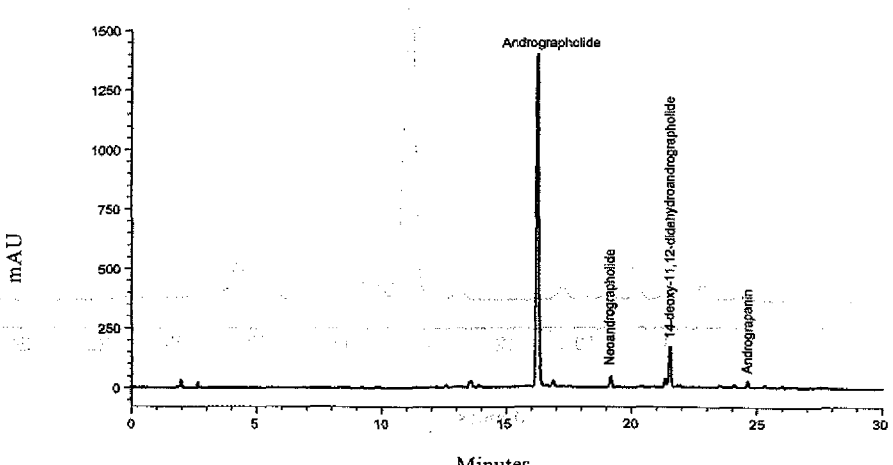
Kalmegh Dry Extract



HPLC Chromatogram of andrographolide IPRS

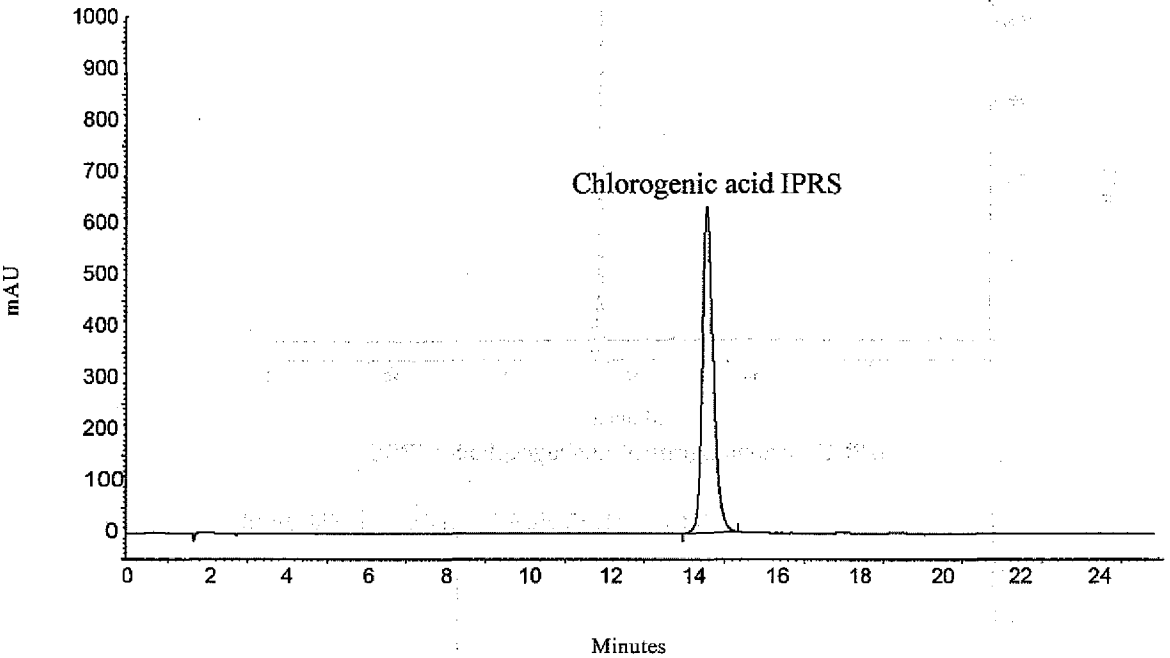


HPLC Chromatogram of 14-deoxy-11, 12-didehydroandrographolide IPRS

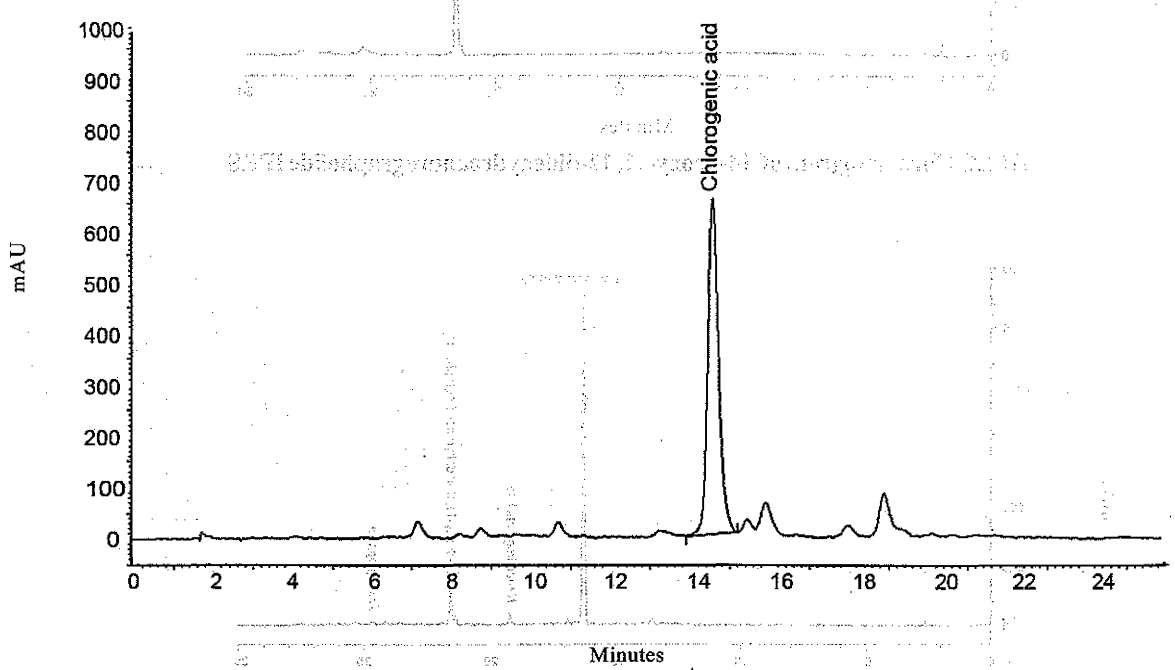


HPLC Chromatogram of Kalmegh Dry Extract

Kasni

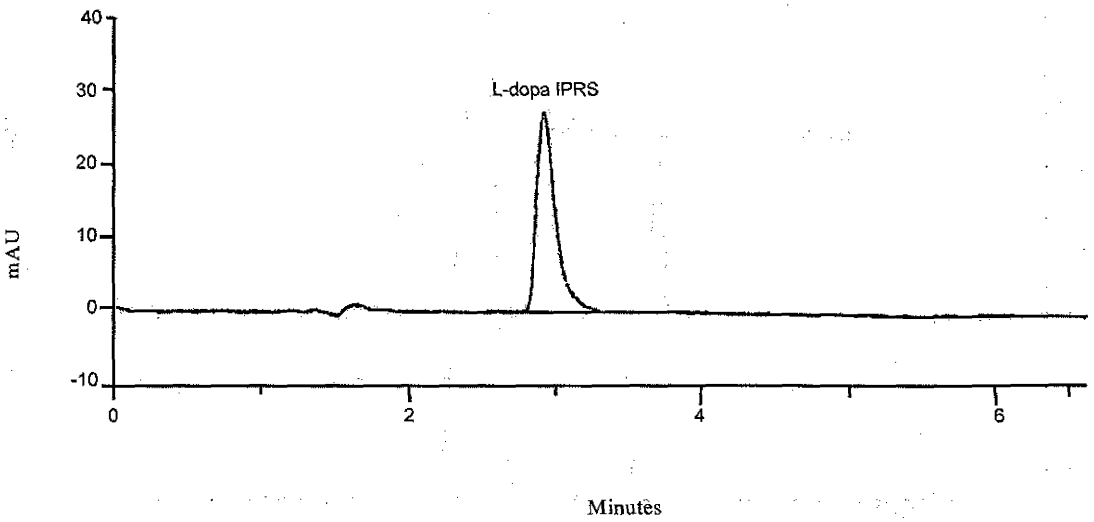


HPLC Chromatogram of Chlorogenic acid IPRS

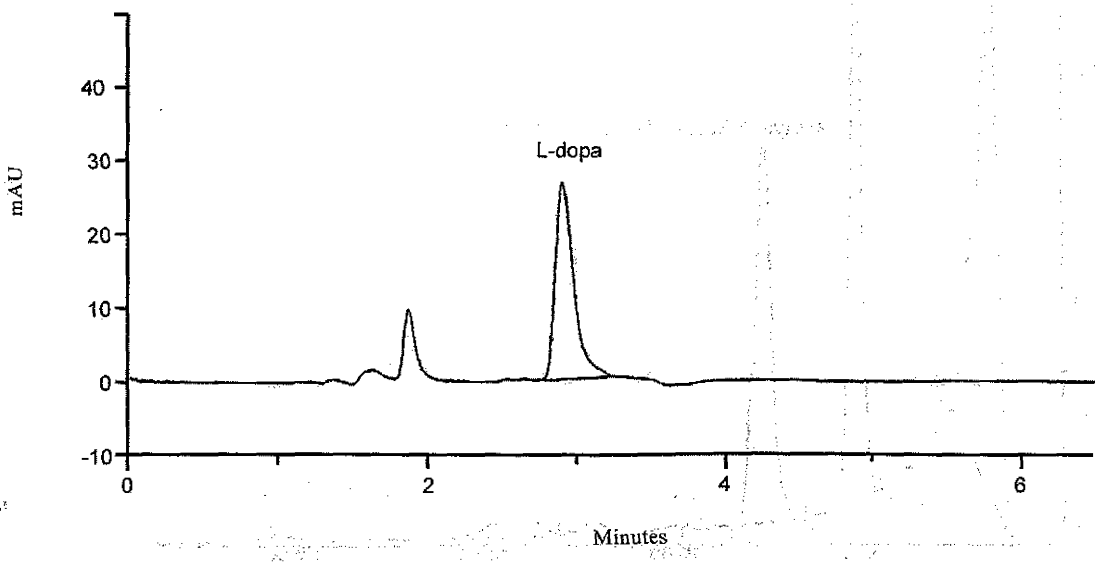


HPLC Chromatogram of Kasni

Kaunch

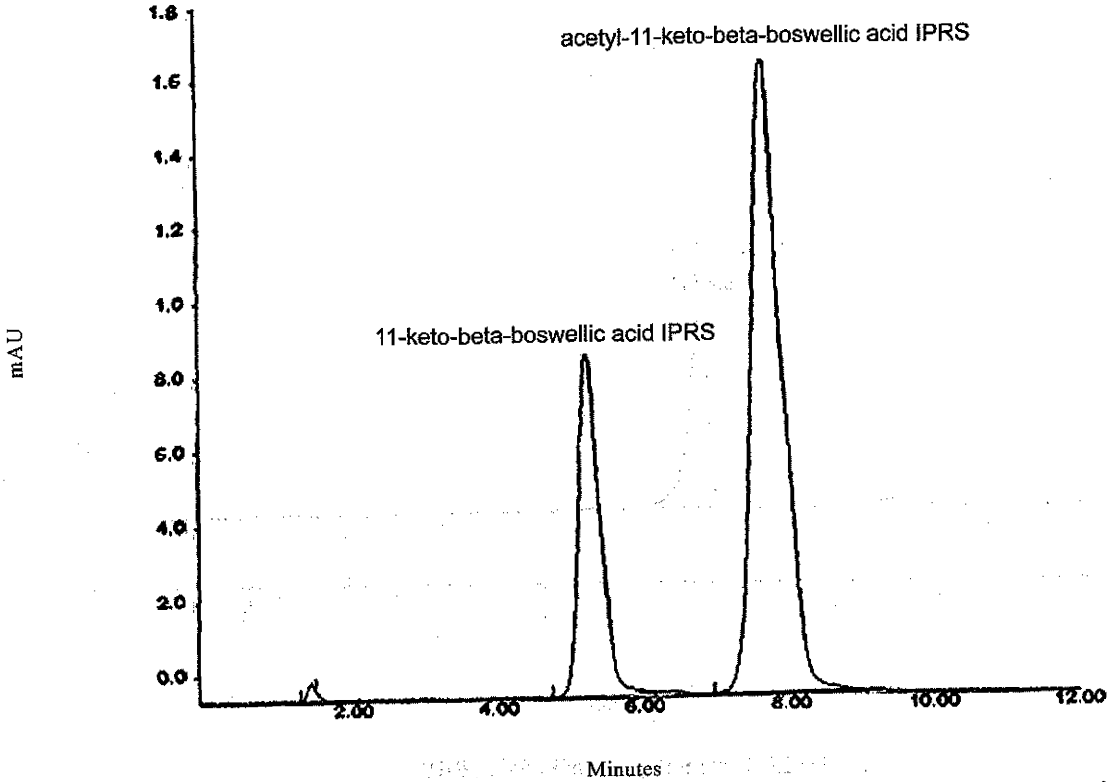


HPLC Chromatogram of L-dopa IPRS

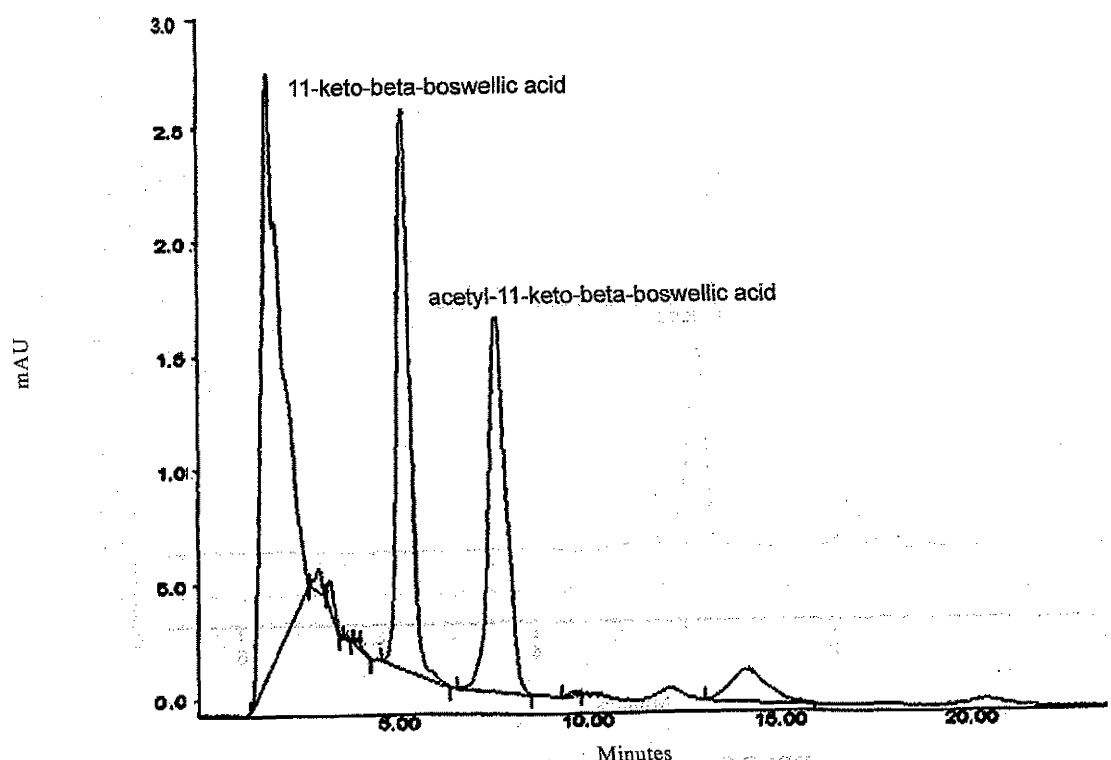


HPLC Chromatogram of Kaunch

Kunduru

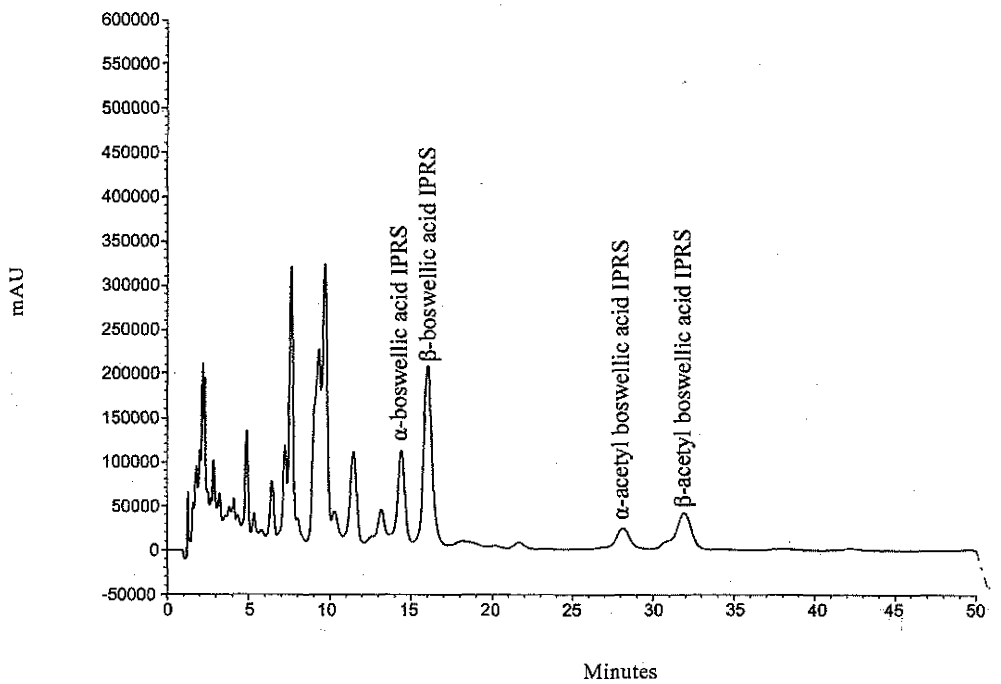


HPLC Chromatogram of 11-keto-beta-boswellic acid IPRS and acetyl-11-keto-beta-boswellic acid IPRS

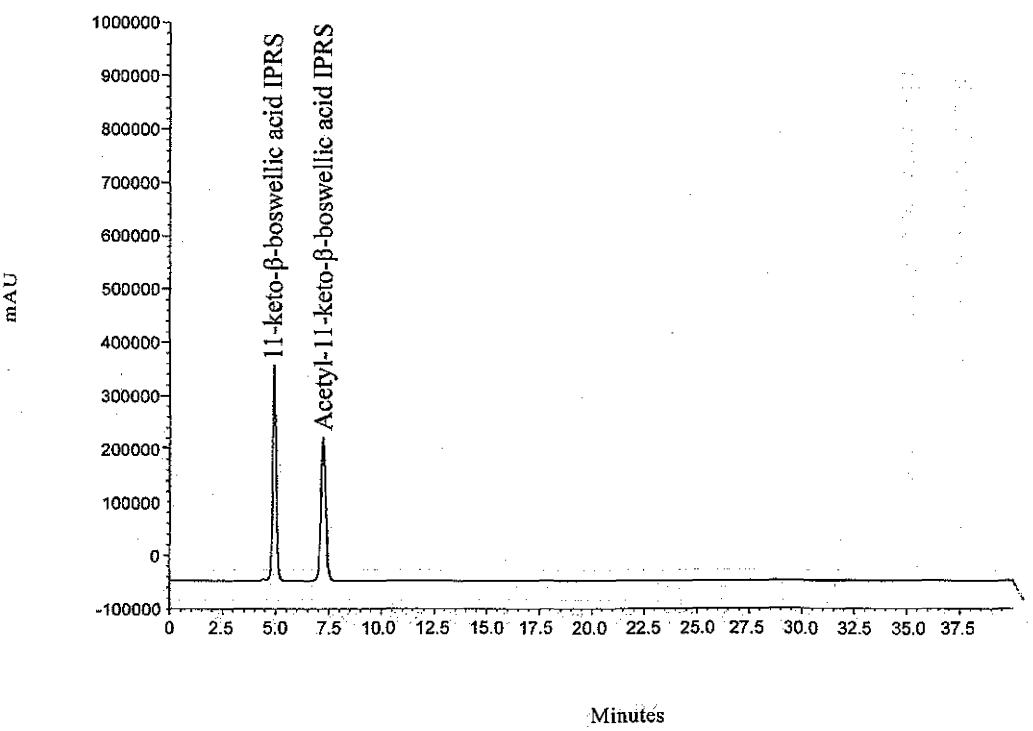


HPLC Chromatogram of Kunduru

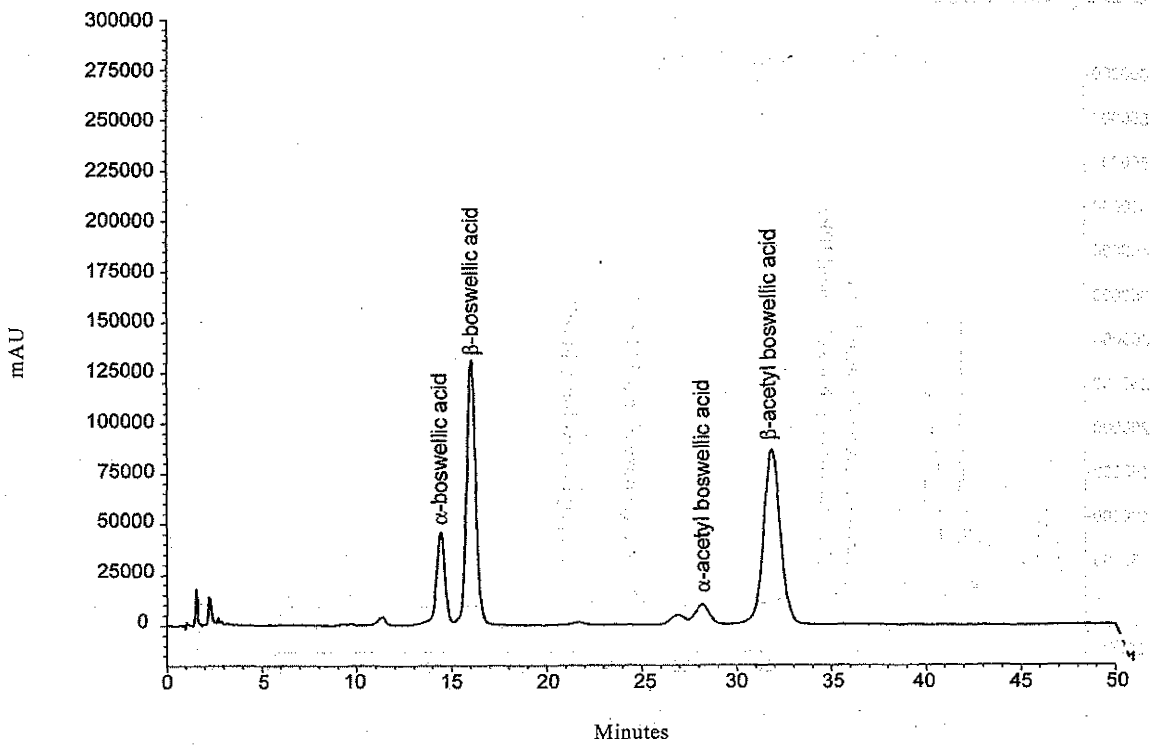
Kunduru Dry Extract



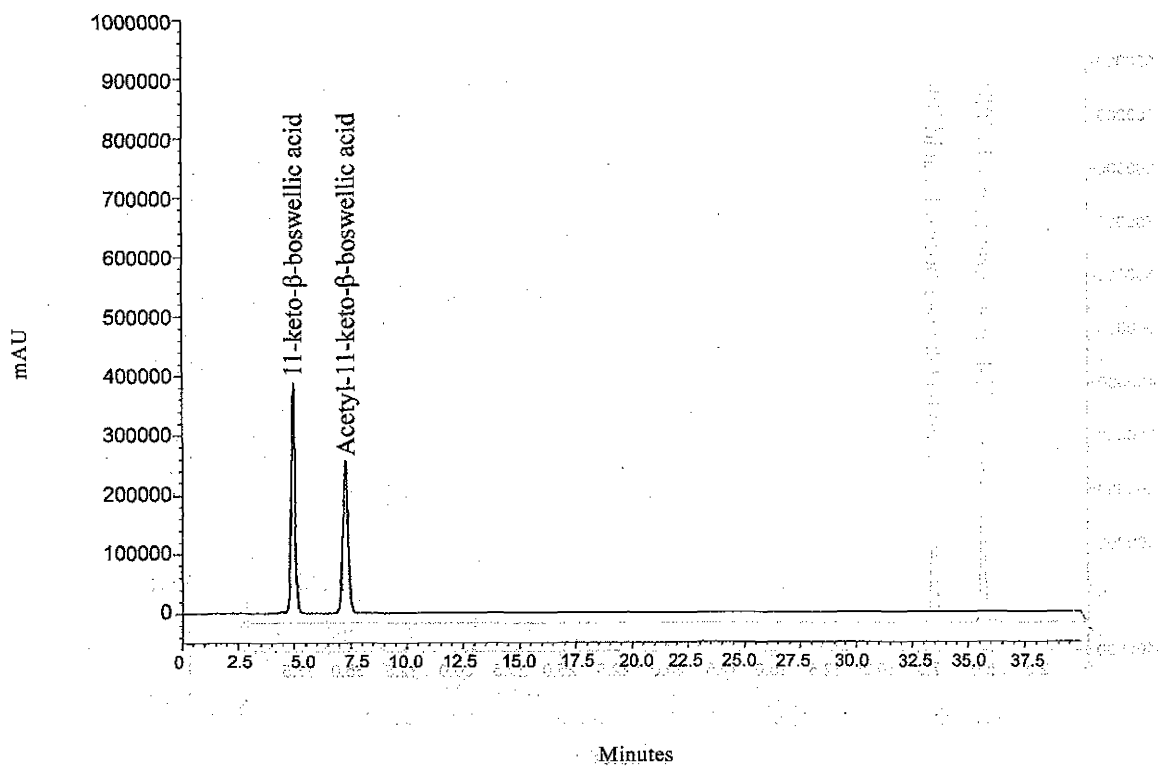
HPLC Chromatogram of α and β beta-boswellic IPRS and α and β boswellic acid IPRS at 205 nm acid



HPLC Chromatogram of 11-keto-β beta-boswellic IPRS and acetyl-11-keto-β boswellic acid IPRS at 247 nm acid

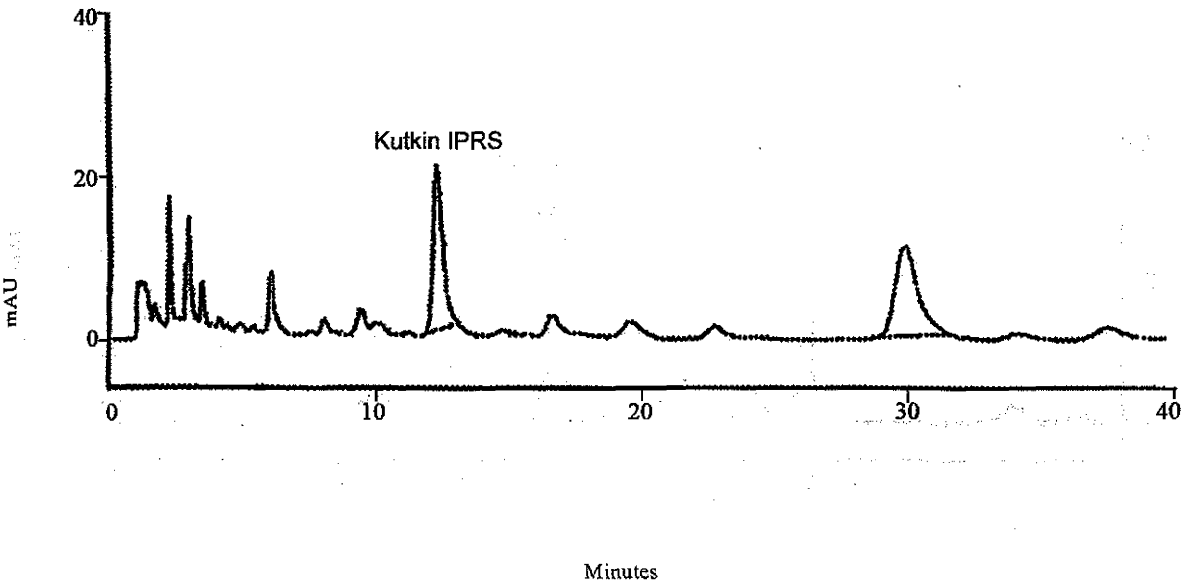


HPLC Chromatogram of Kunduru Dry Extract at 205 nm

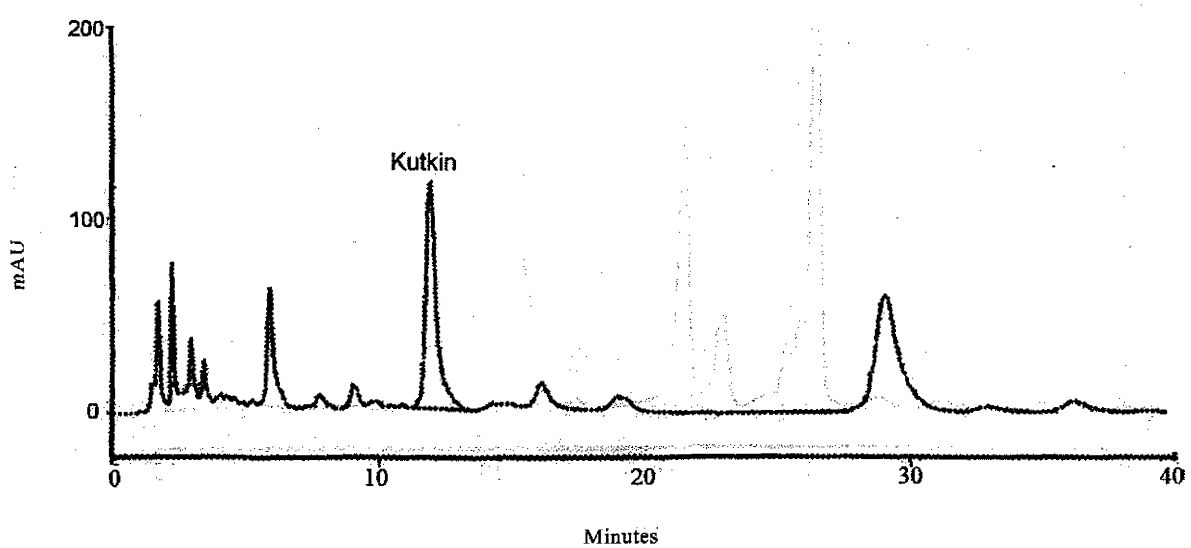


HPLC Chromatogram of Kunduru Dry Extract at 247 nm

Kutki

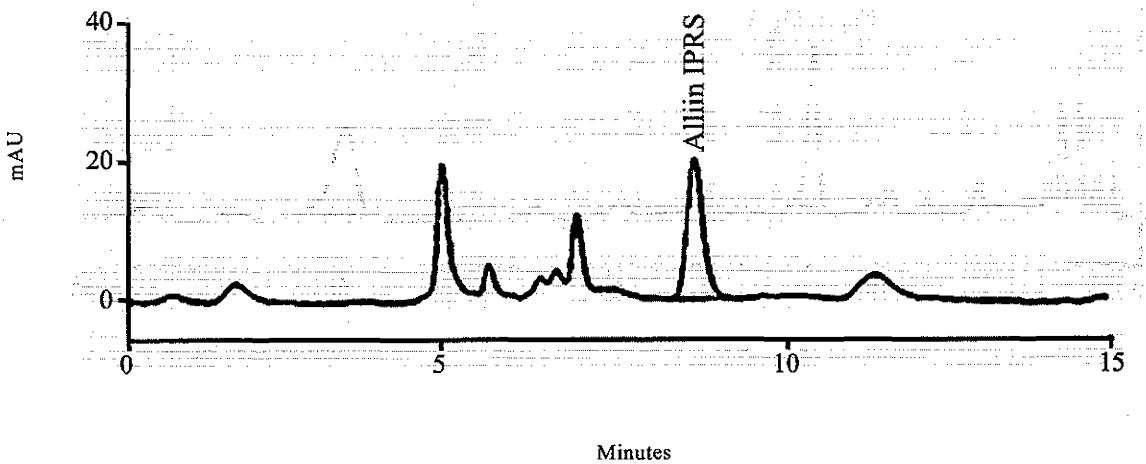


HPLC Chromatogram of kutkin IPRS

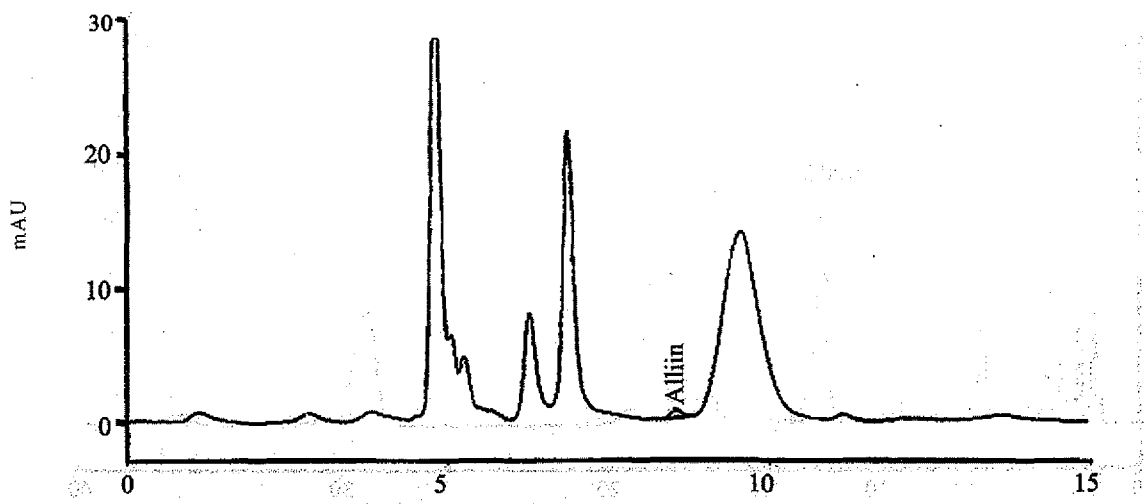


HPLC Chromatogram of Kutki

Lasuna

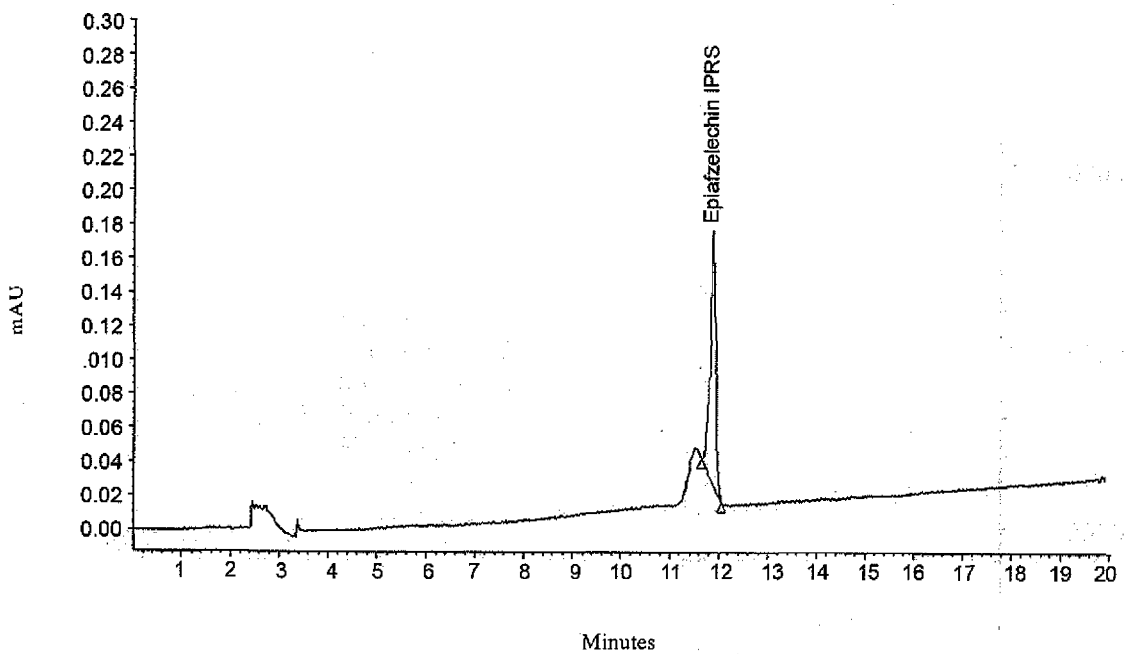


HPLC Chromatogram of alliin IPRS

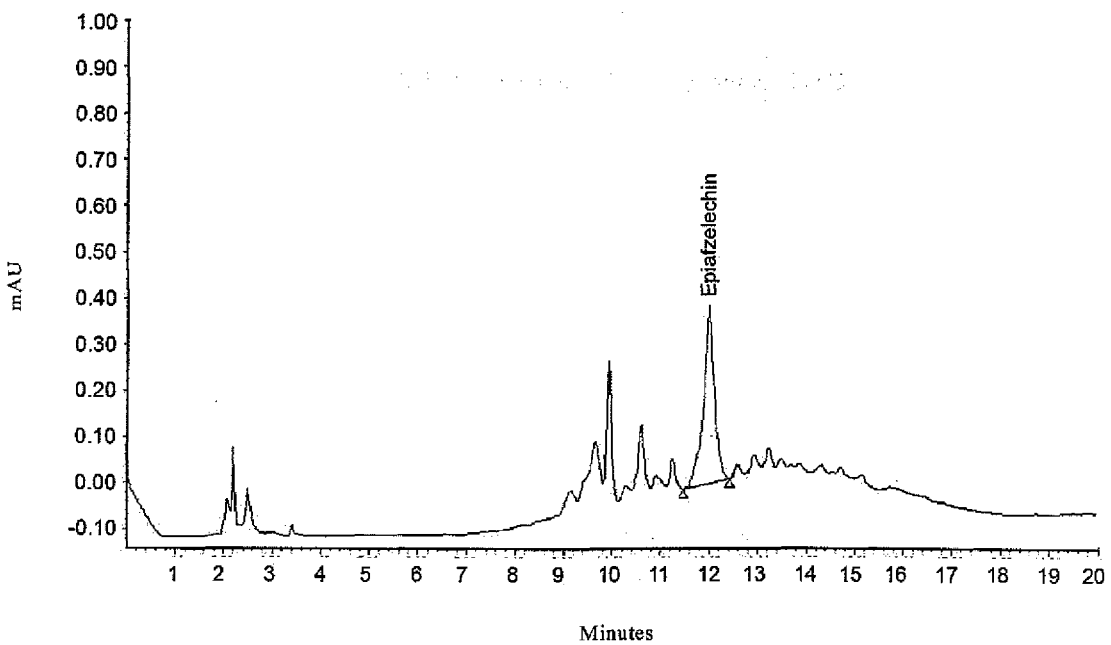


HPLC Chromatogram of Lasuna

Lodhra

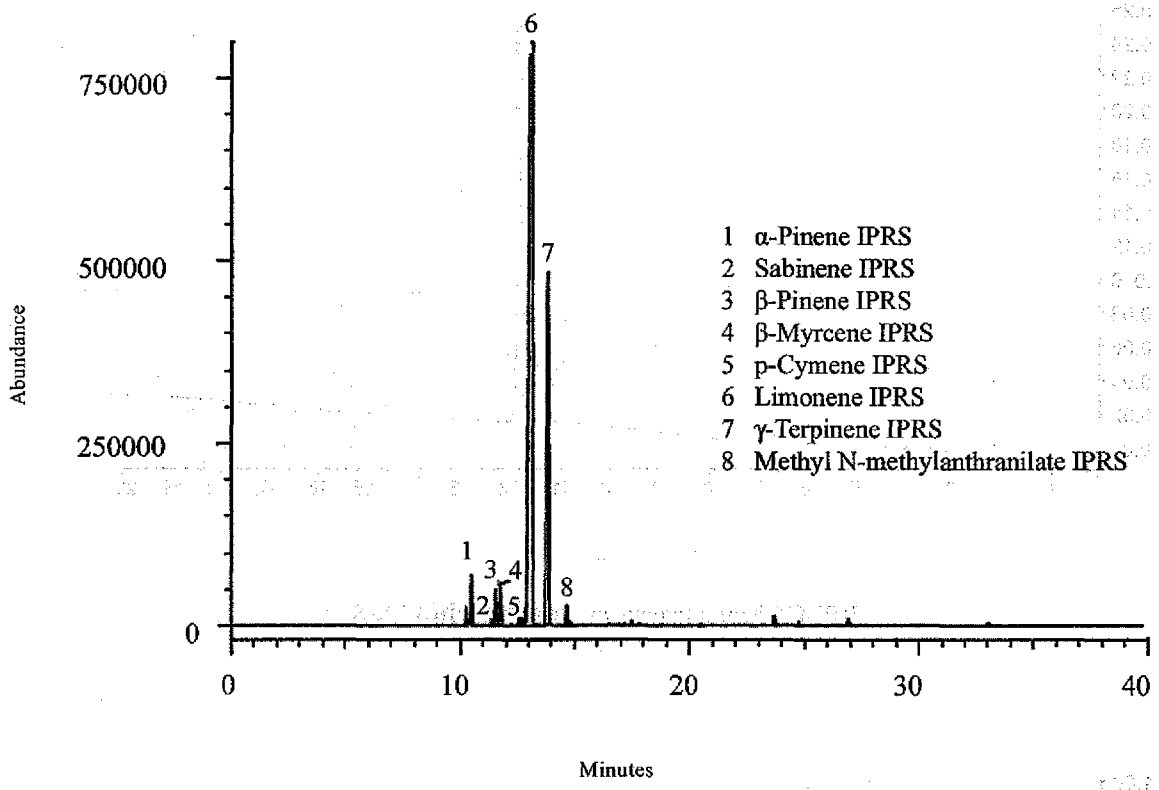


HPLC Chromatogram of epiafzelechin IPRS



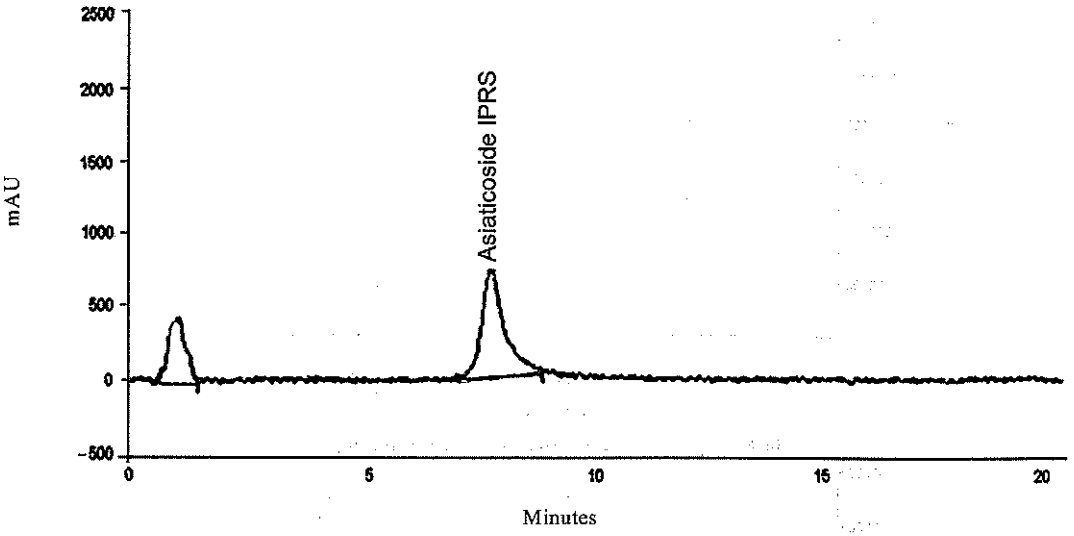
HPLC Chromatogram of Lodhra

Mandarin Oil

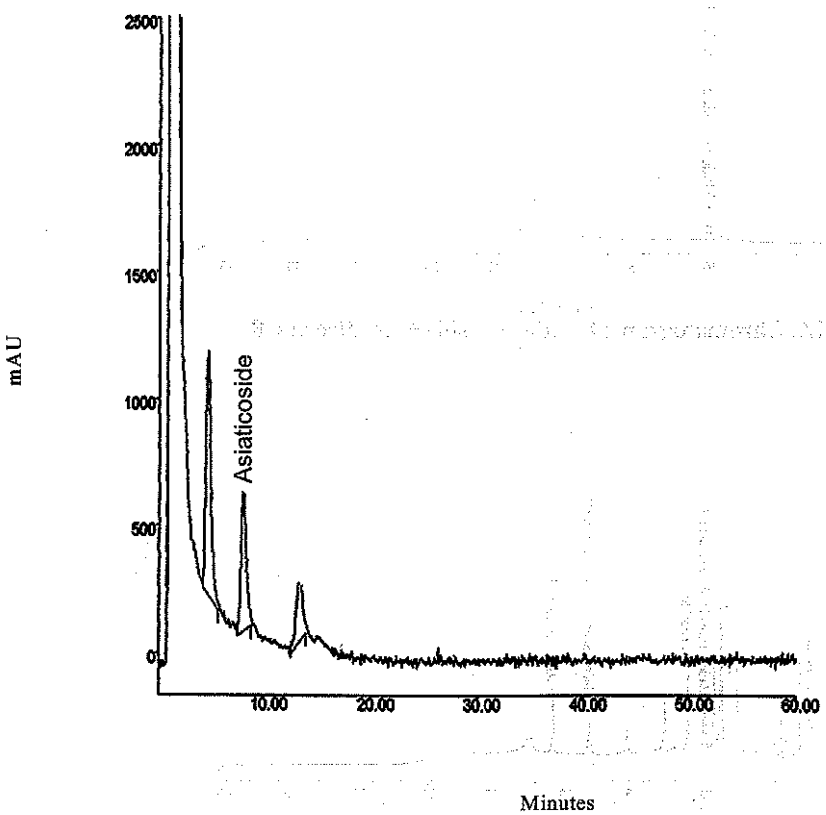


Gas Chromatogram of Mandarin Oil IPRS

Mandukaparni

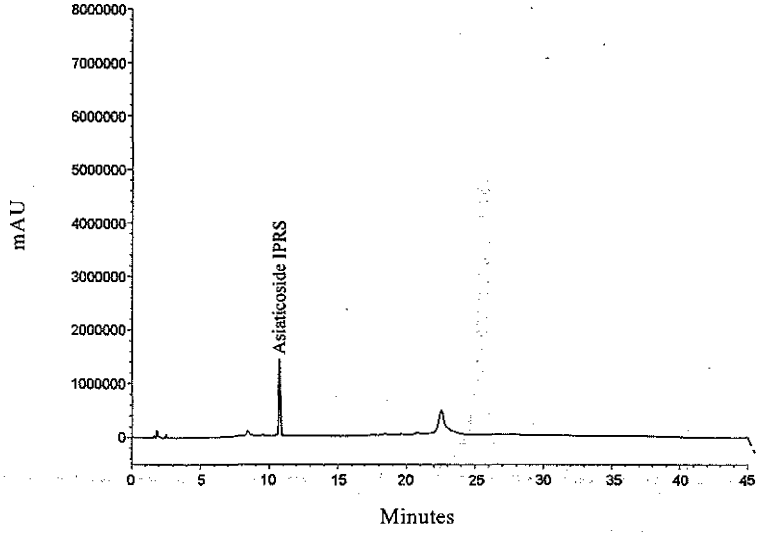


HPLC Chromatogram of asiaticoside IPRS

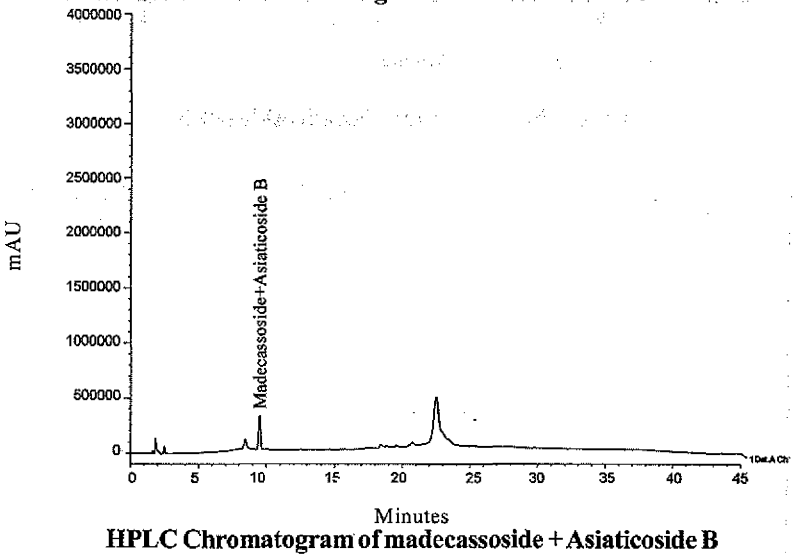


HPLC Chromatogram of Mandukaparni

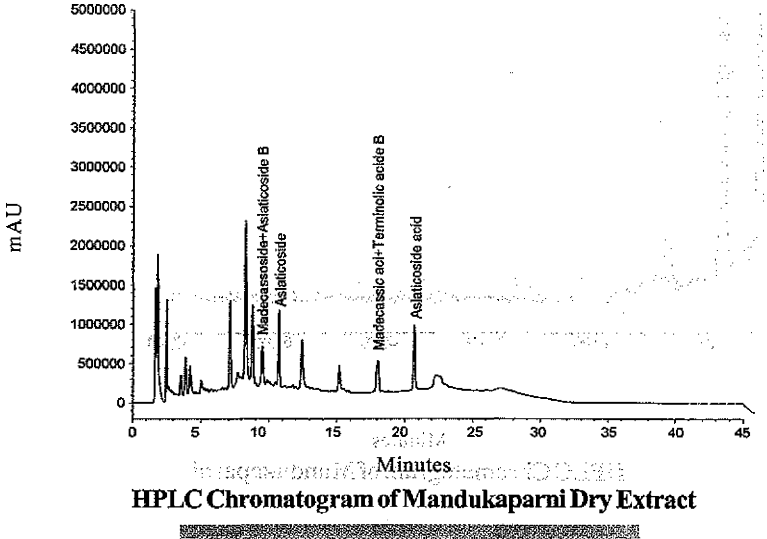
Mandukaparni Dry Extract



HPLC Chromatogram of asiaticoside IPRS

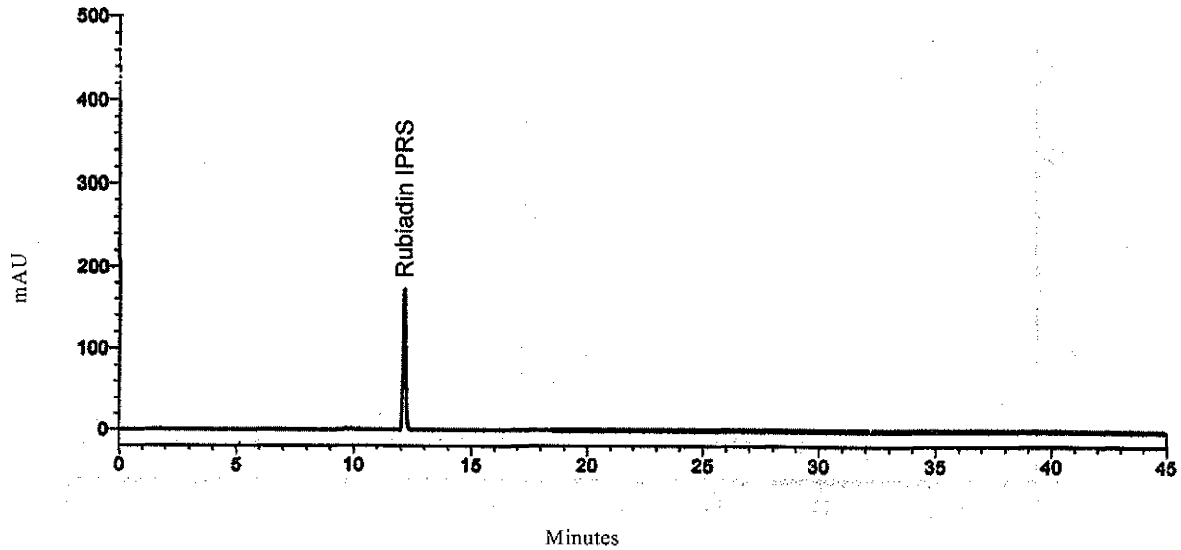


HPLC Chromatogram of madecassoside + Asiaticoside B

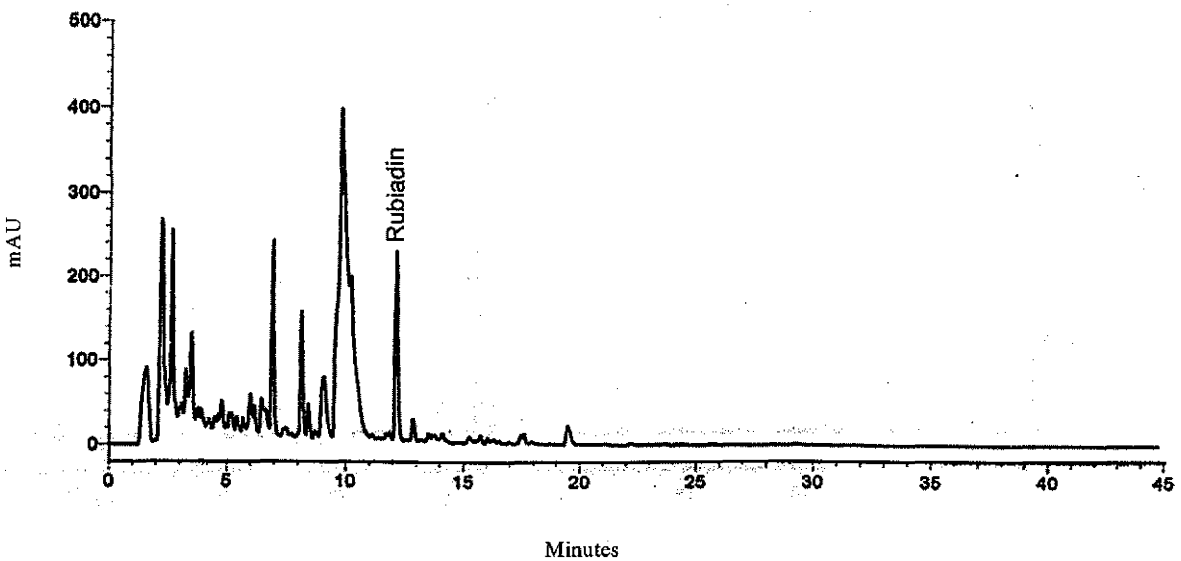


HPLC Chromatogram of Mandukaparni Dry Extract

Manjistha

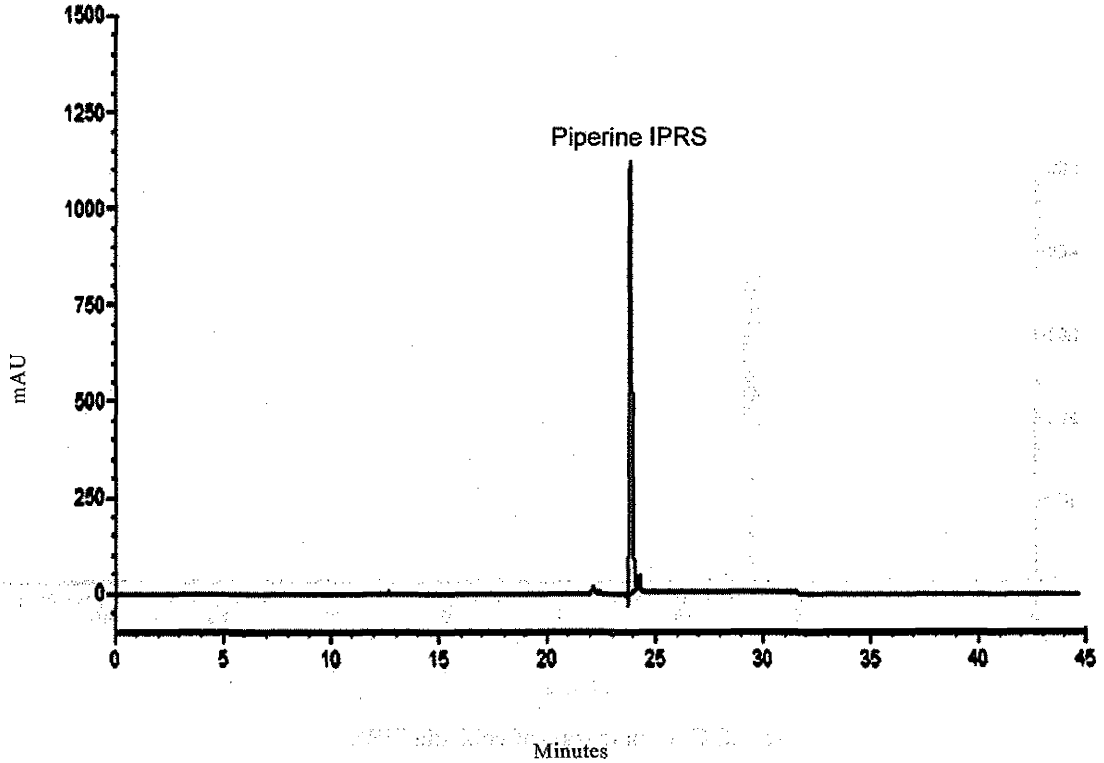


HPLC Chromatogram of rubiadin IPRS

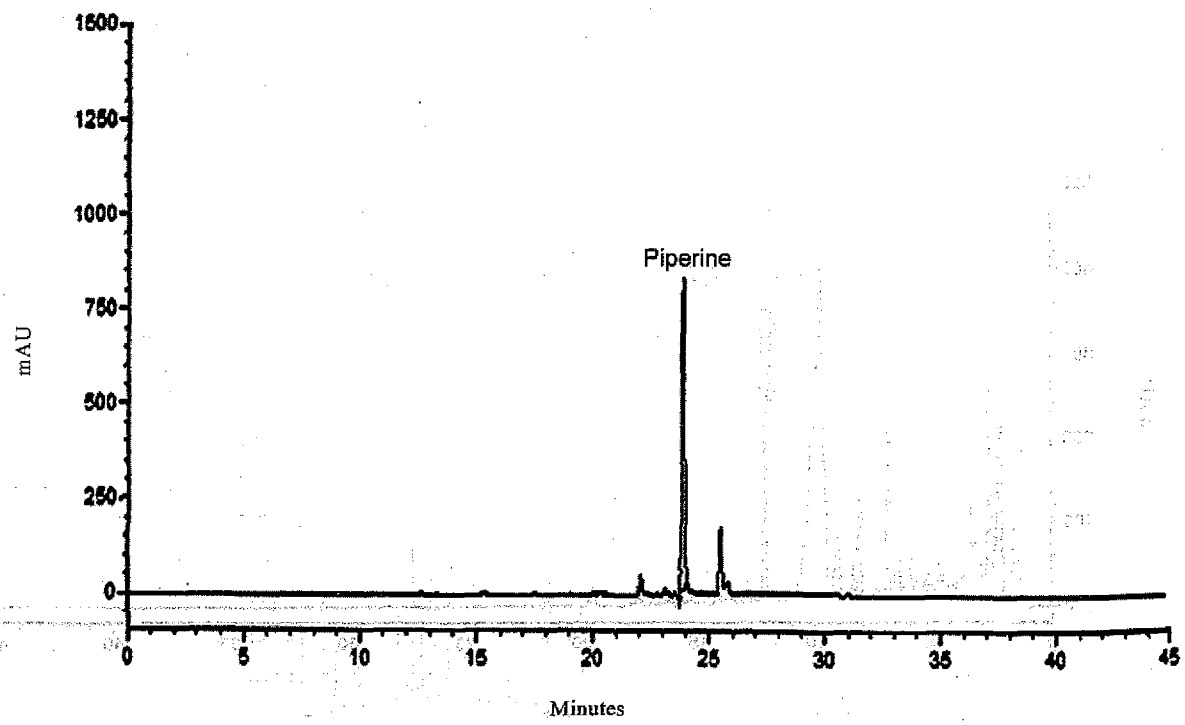


HPLC Chromatogram of Manjistha

Maricha

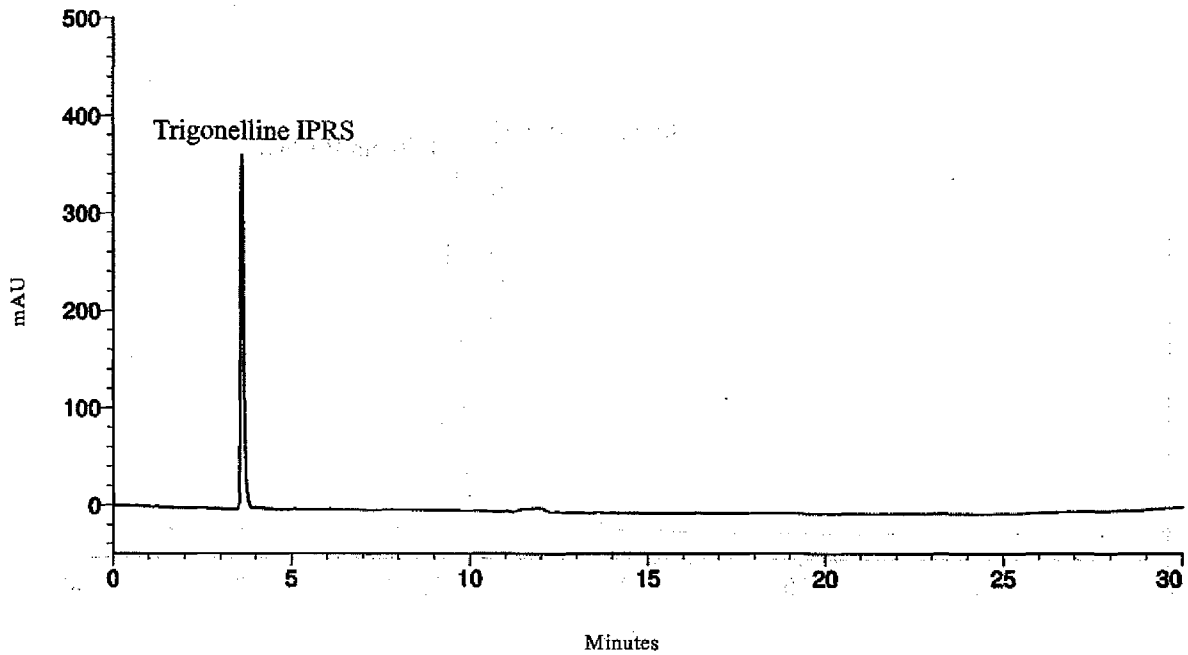


HPLC Chromatogram of piperine IPRS

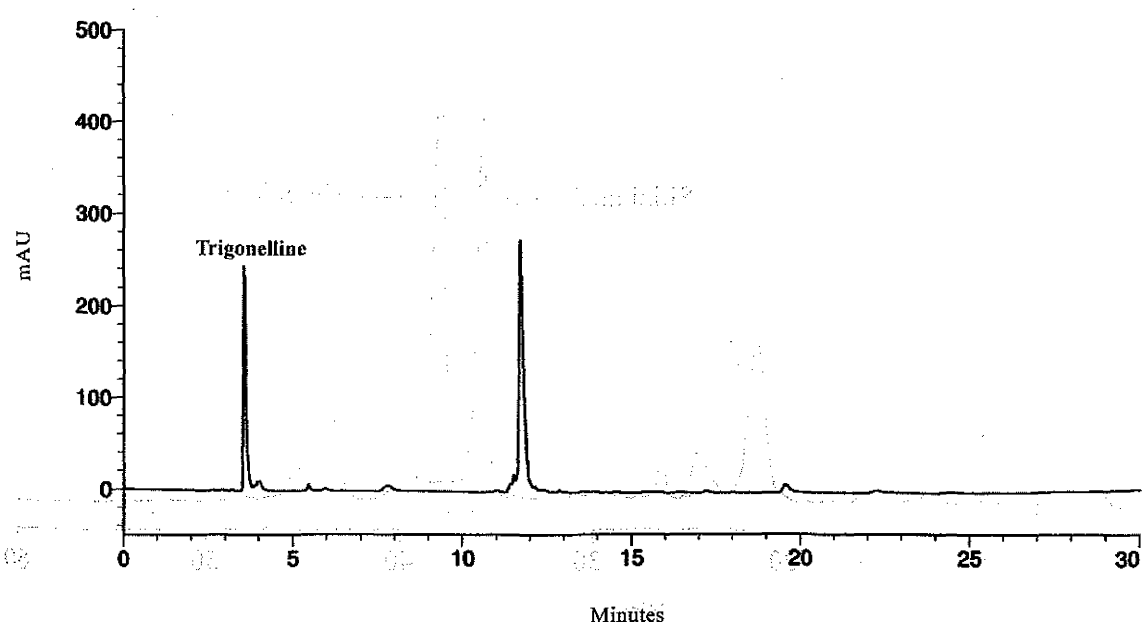


HPLC Chromatogram of Maricha

Methi

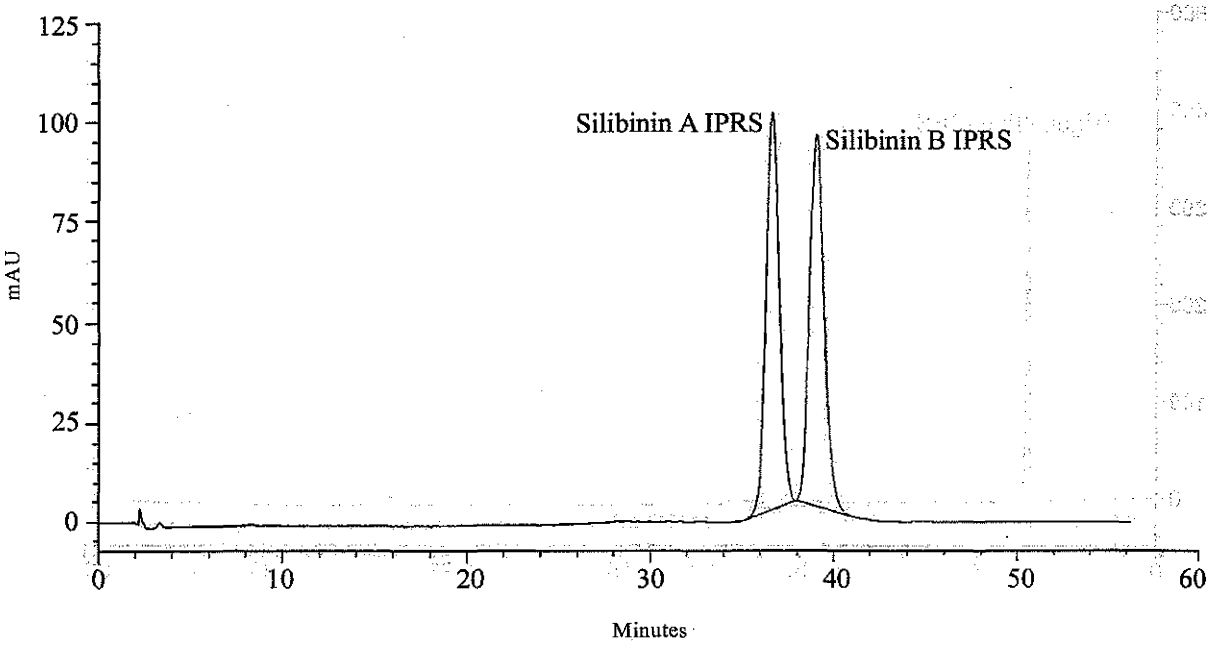


HPLC Chromatogram of trigonelline IPRS

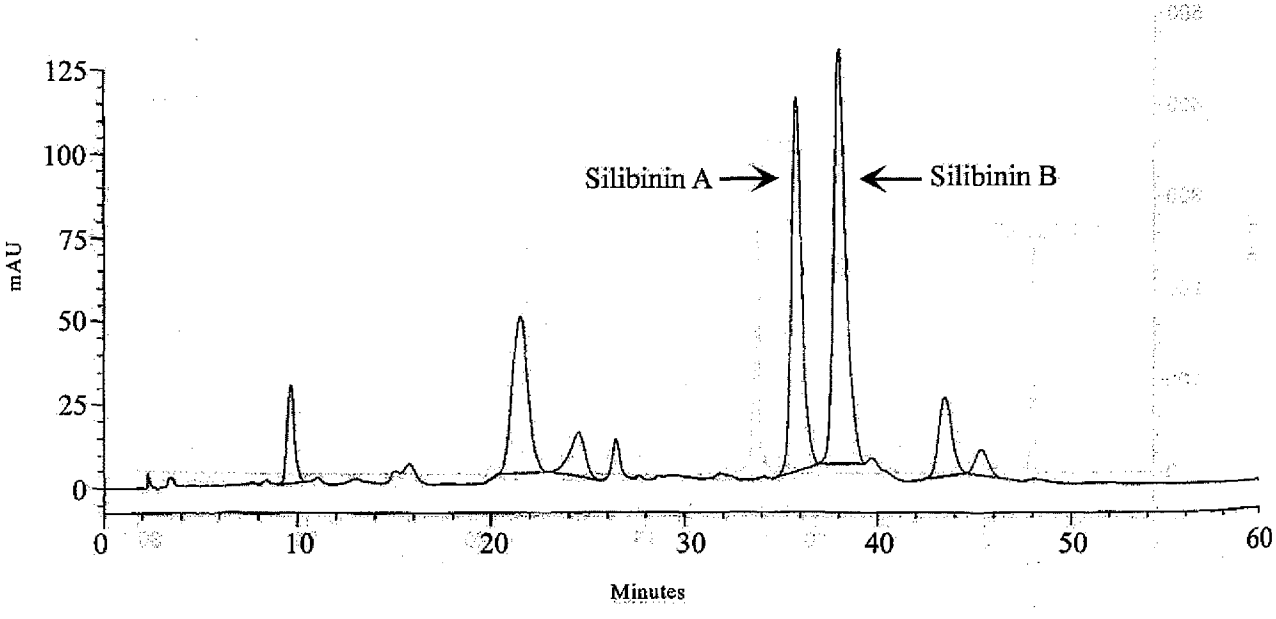


HPLC Chromatogram of Methi

Milk Thistle

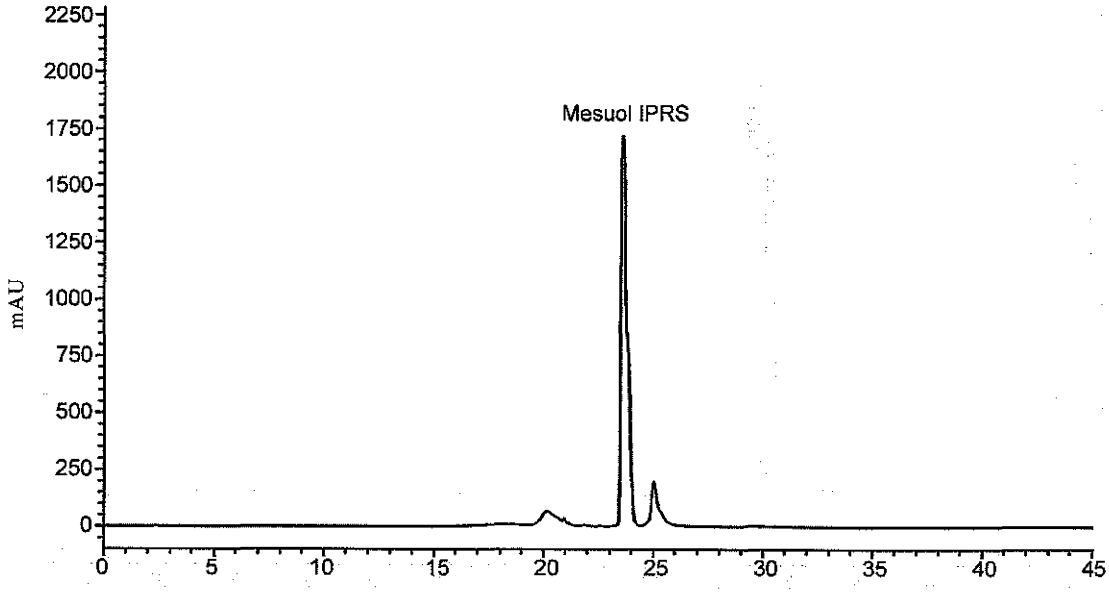


HPLC Chromatogram of silibinin A IPRS and silibinin B IPRS

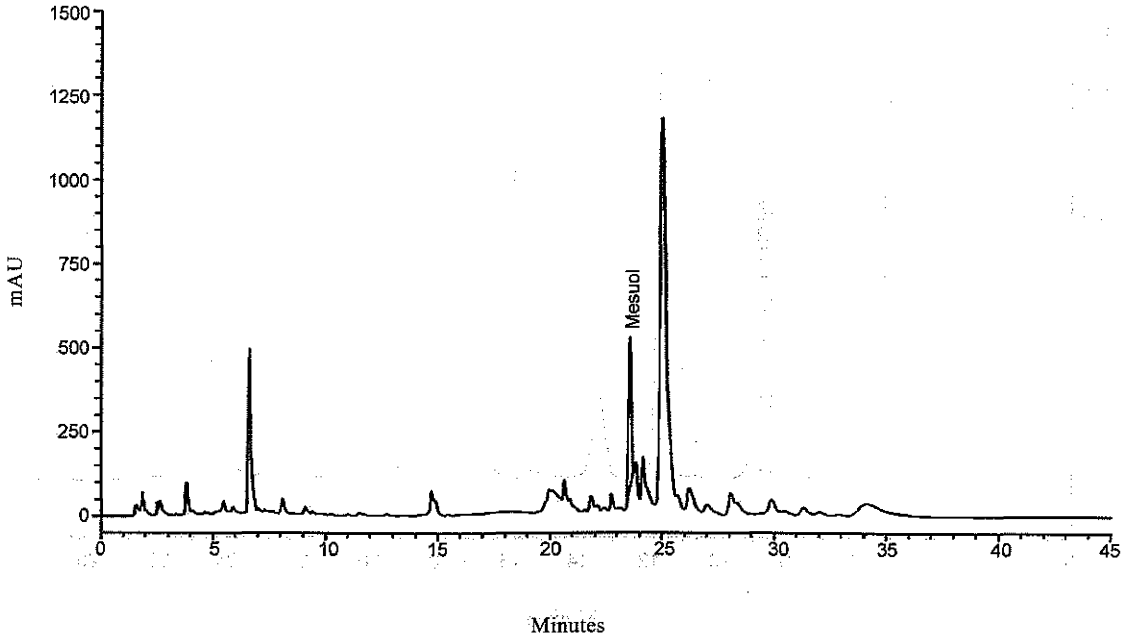


HPLC Chromatogram of Milk Thistle Dry Extract

Nagakesar

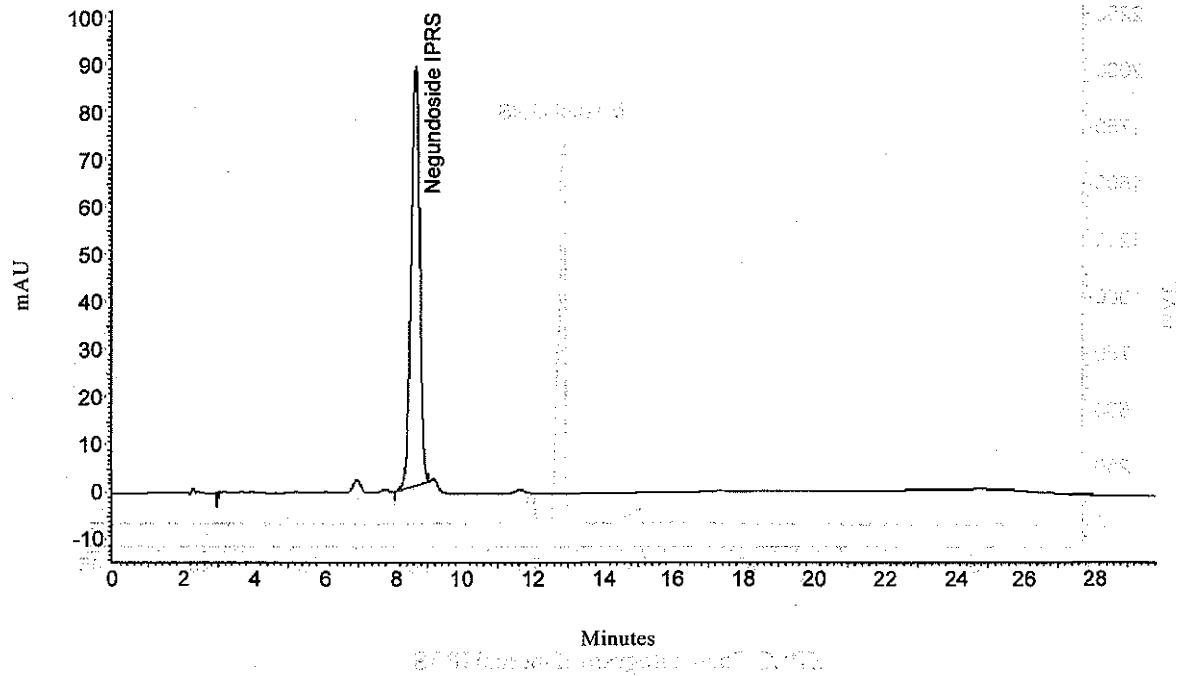


HPLC Chromatogram of mesuol IPRS

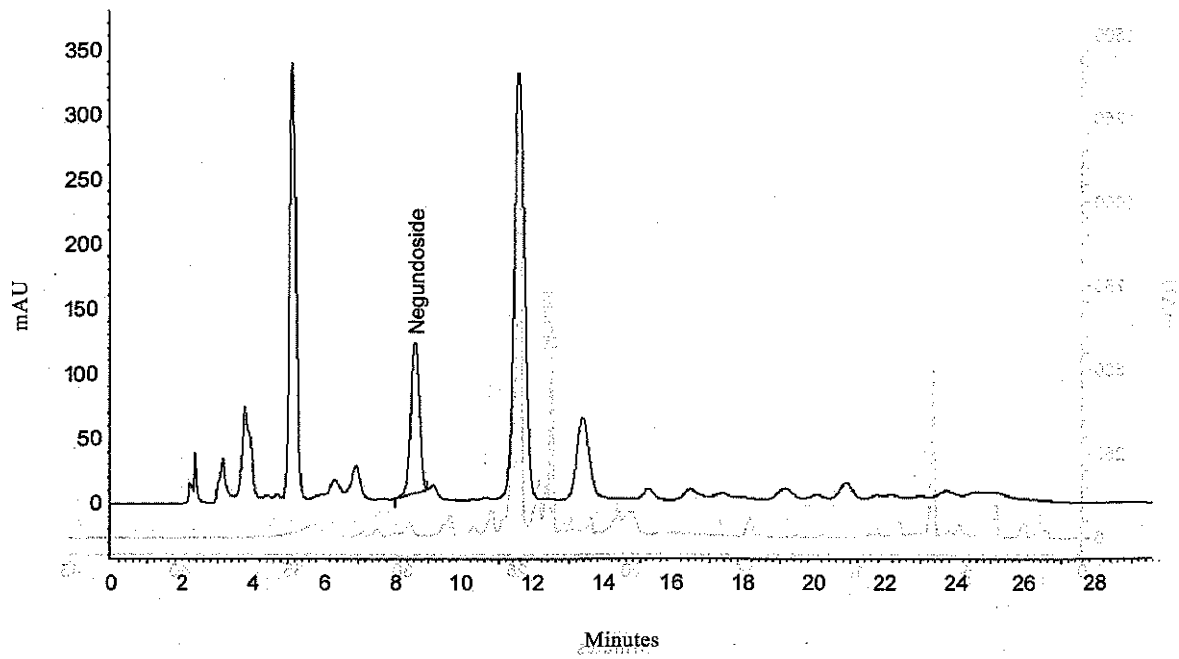


HPLC Chromatogram of Nagakesar

Nirgundi

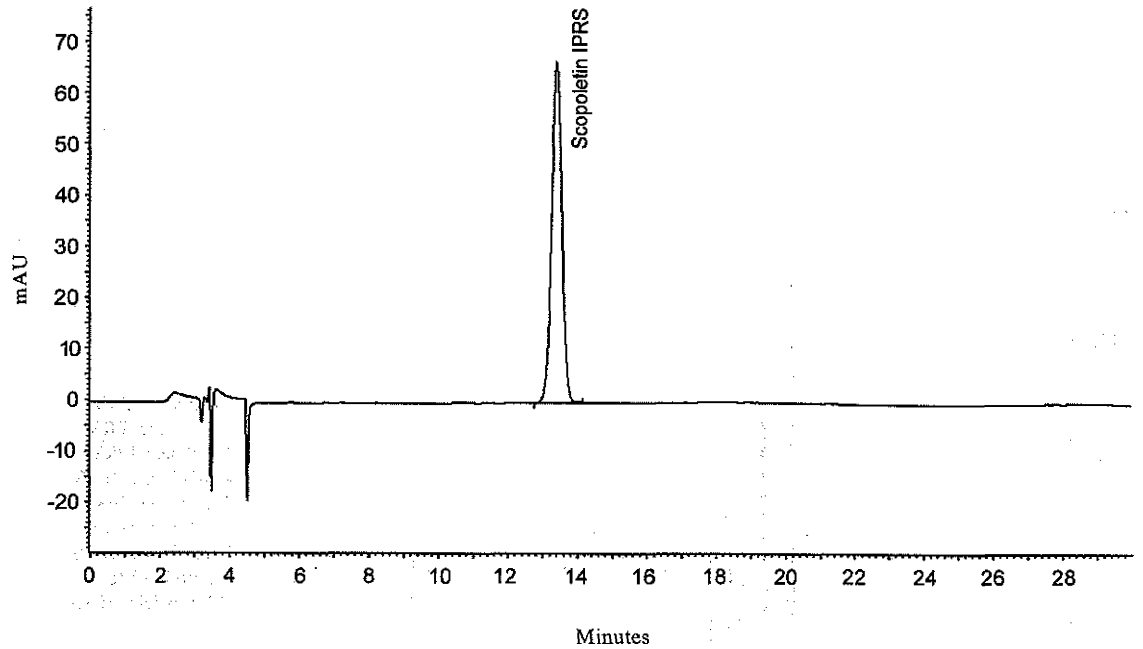


HPLC Chromatogram of negundoside IPRS

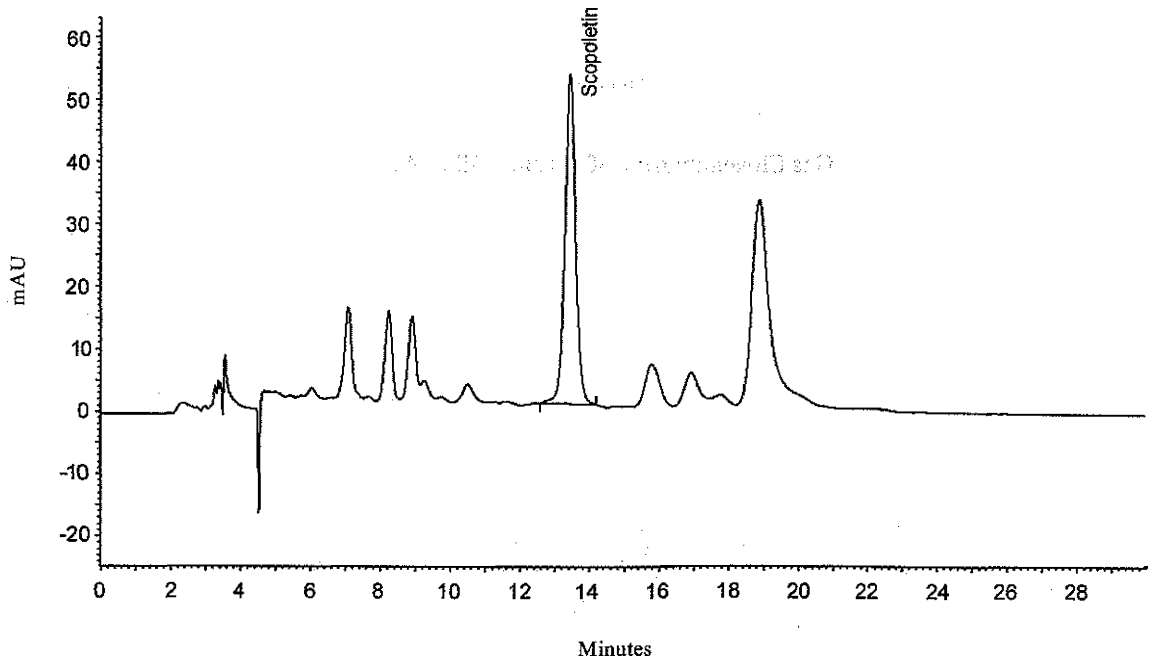


HPLC Chromatogram of Nirgundi

Noni

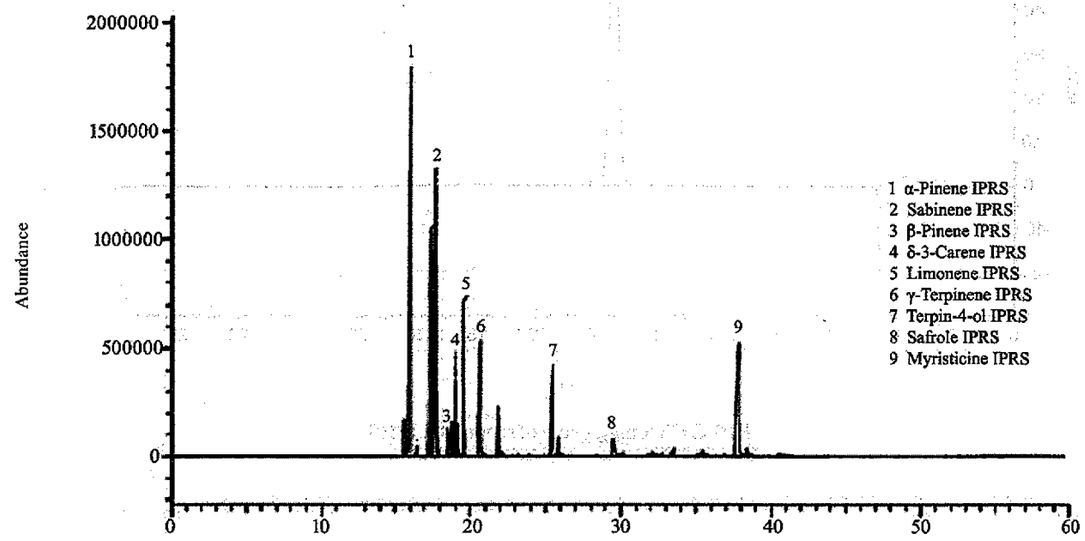


HPLC Chromatogram of scopoletin IPRS



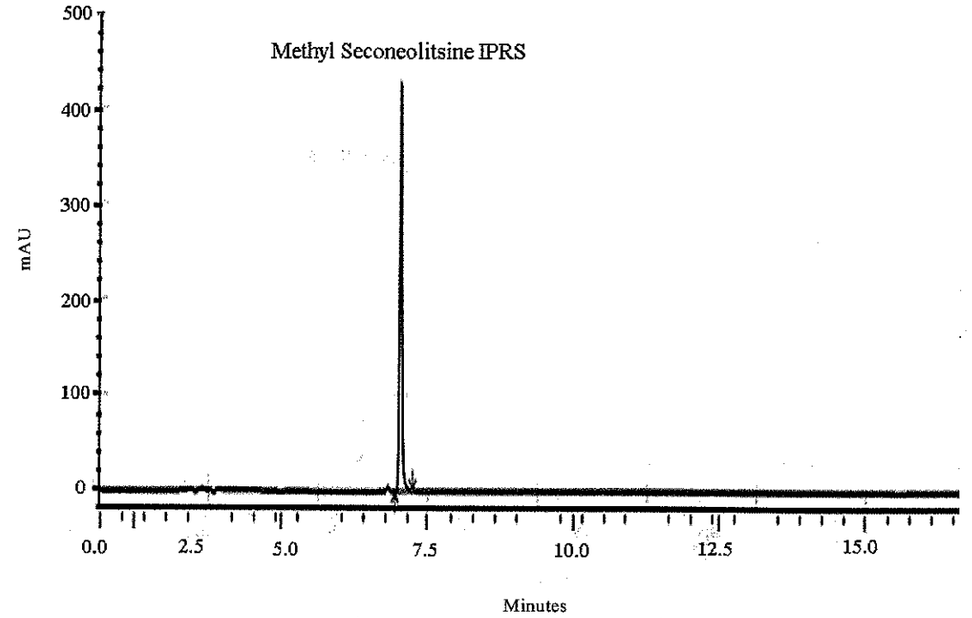
HPLC Chromatogram of Noni

Nutmeg Oil

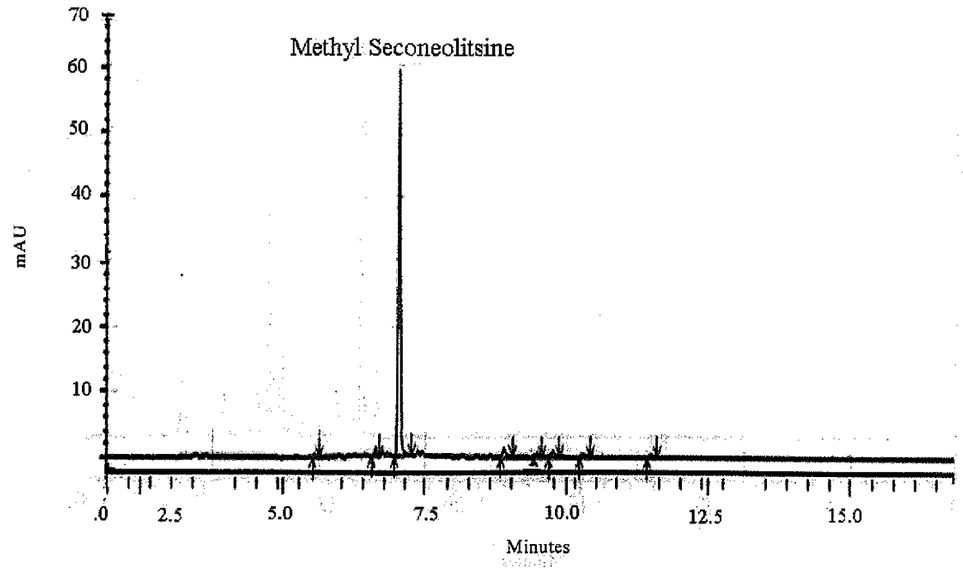


Gas Chromatogram of Nutmeg Oil IPRS

Patha

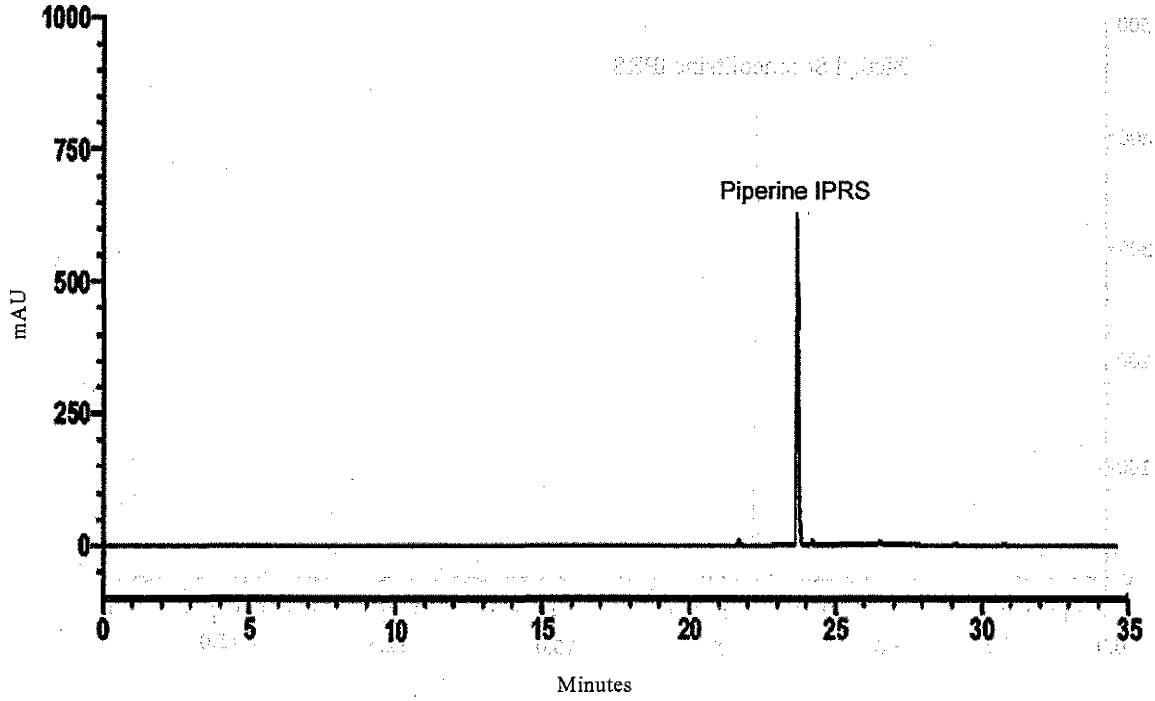


HPLC Chromatogram of methyl seconeolitsine IPRS

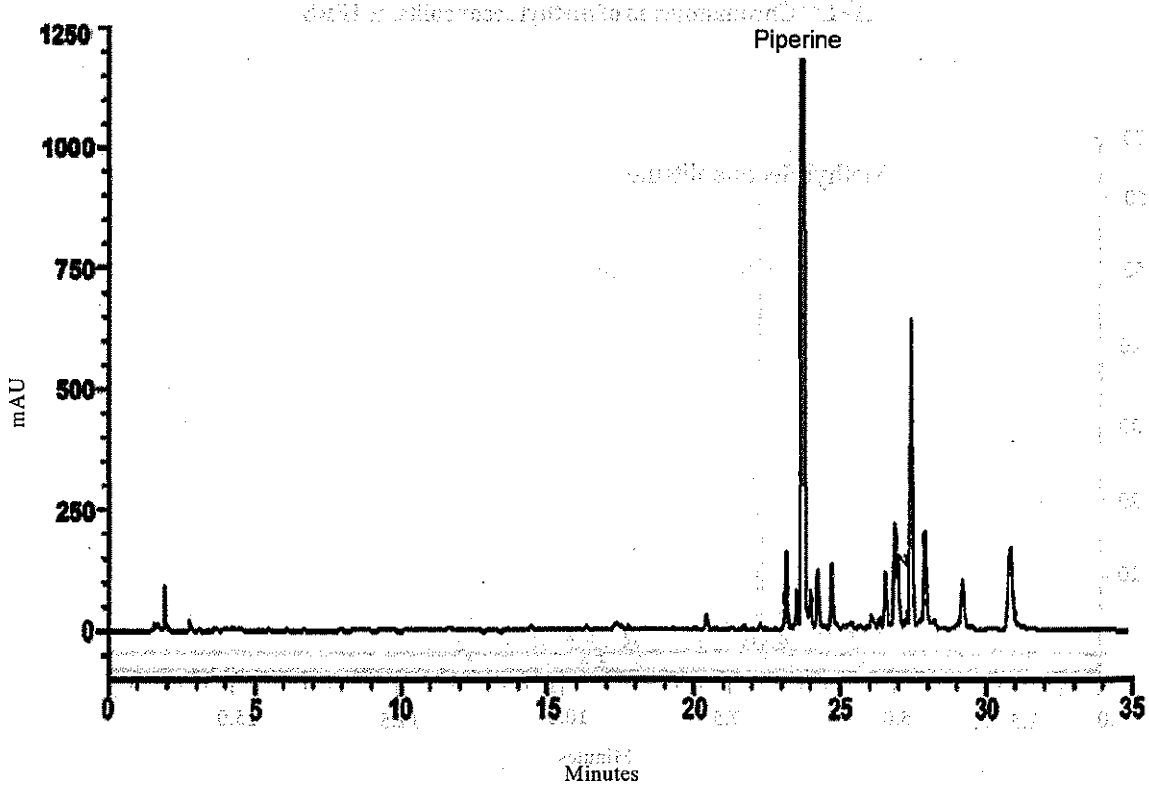


HPLC Chromatogram of Patha

Pippali (Large) and Pippali (Small)

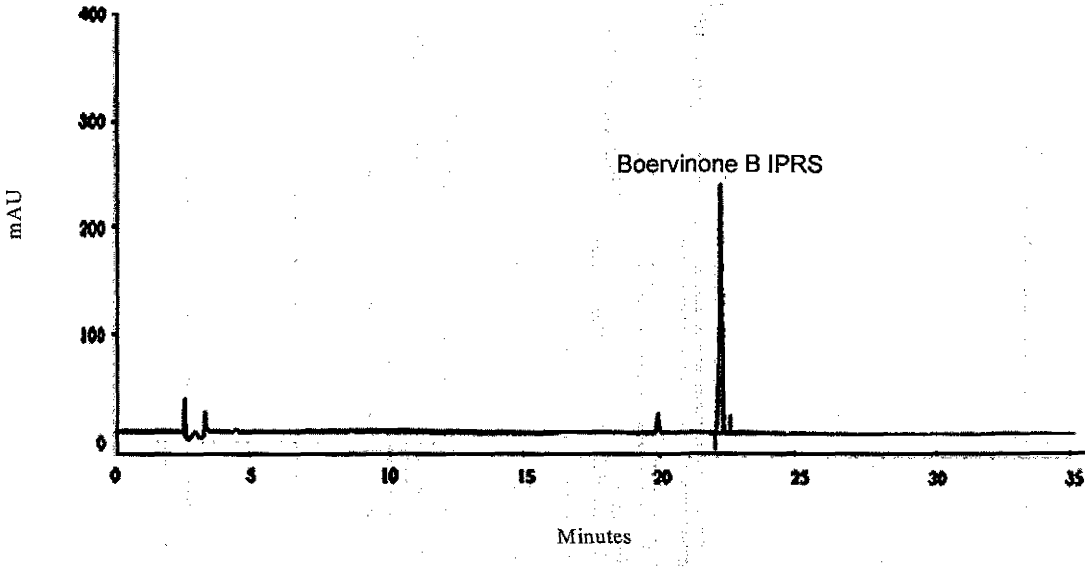


HPLC Chromatogram of piperine IPRS

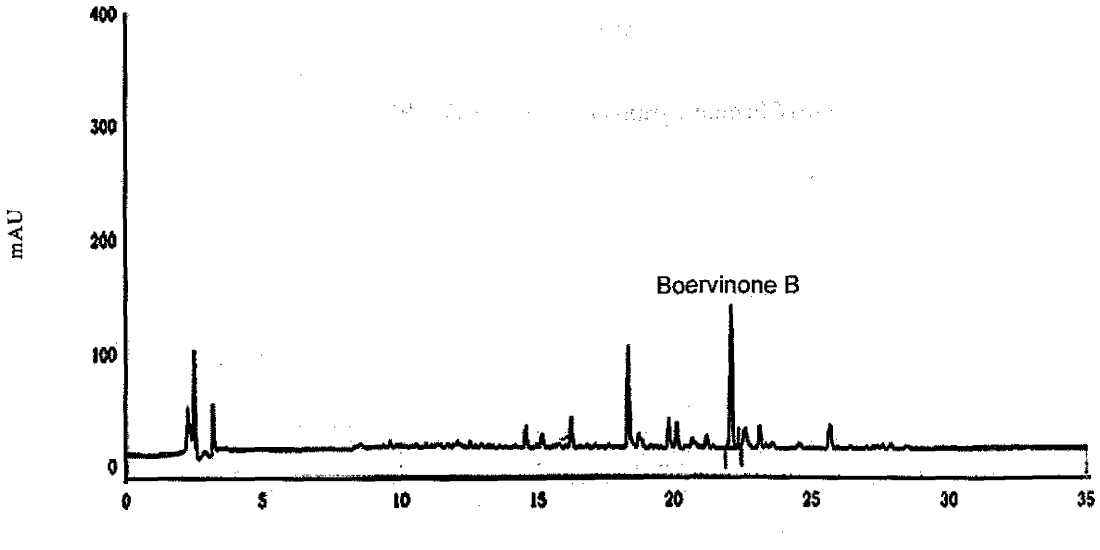


HPLC Chromatogram of Pippali (Large) and Pippali (Small)

Punarnava

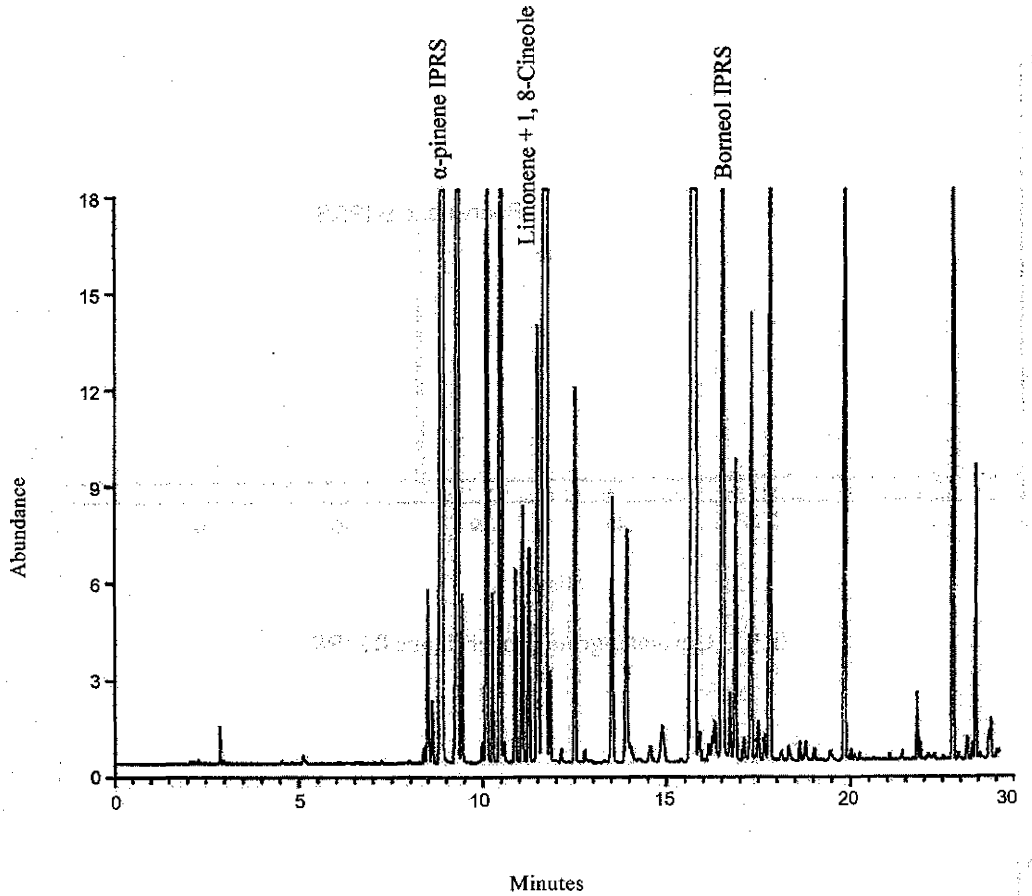


HPLC Chromatogram of boervinone B IPRS



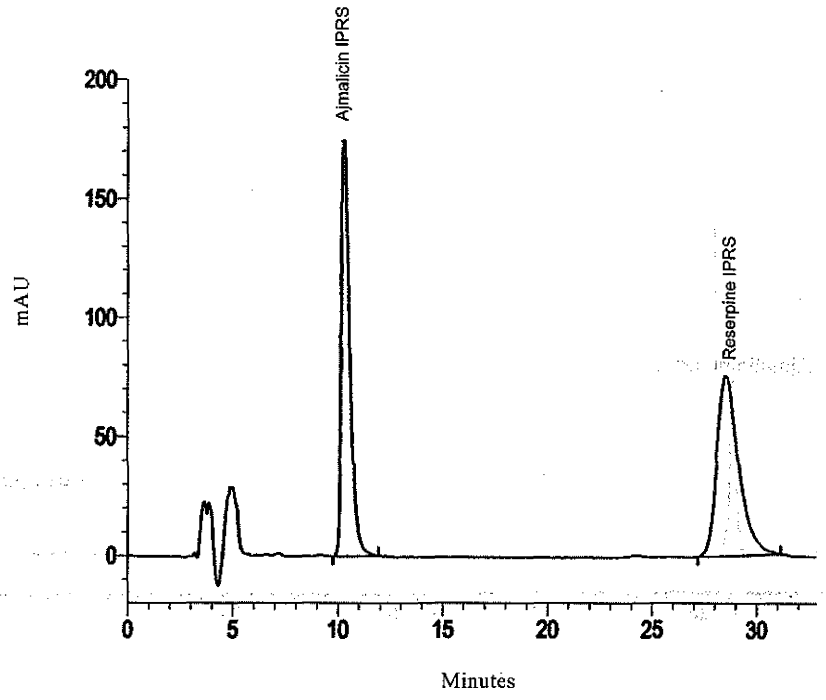
HPLC Chromatogram of Punarnava

Rosemary Oil

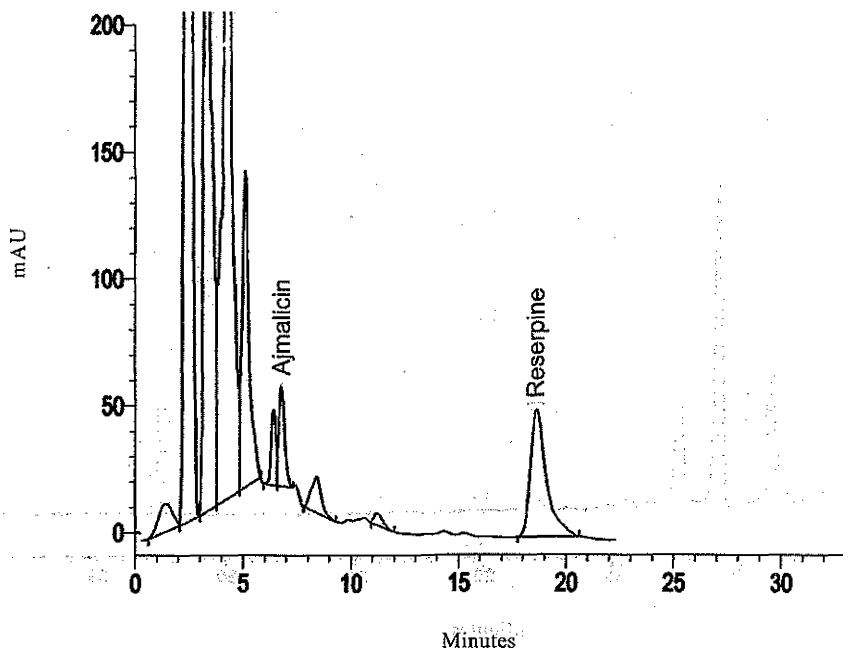


Gas Chromatogram of Rosemary Oil IPRS

Sarpagandha

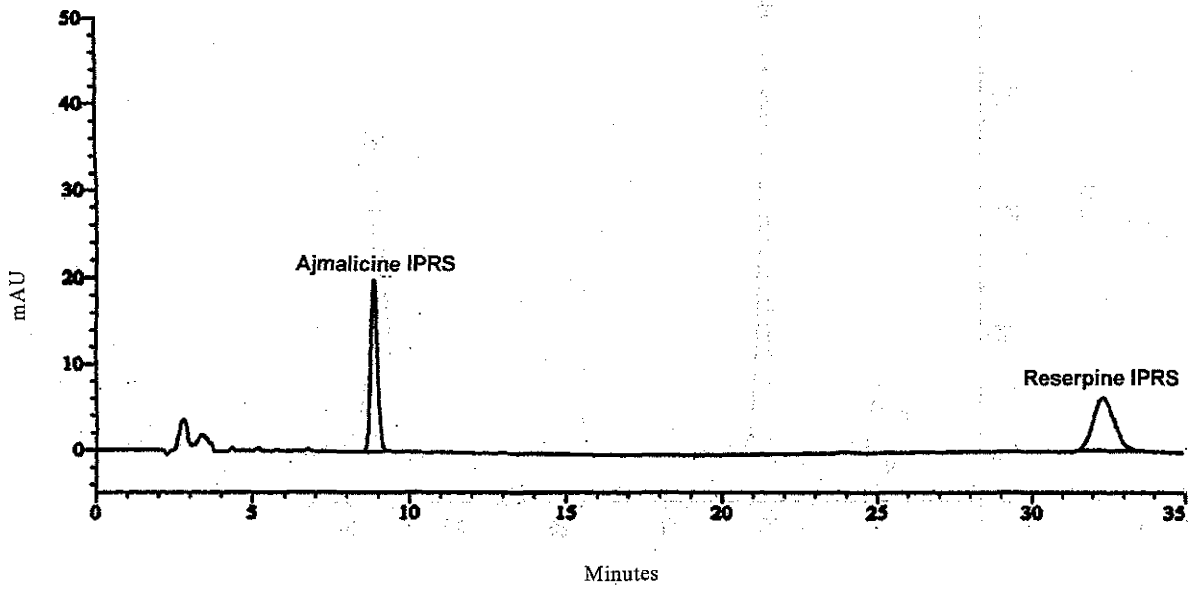


HPLC Chromatogram of ajmalicin IPRS and reserpine IPRS

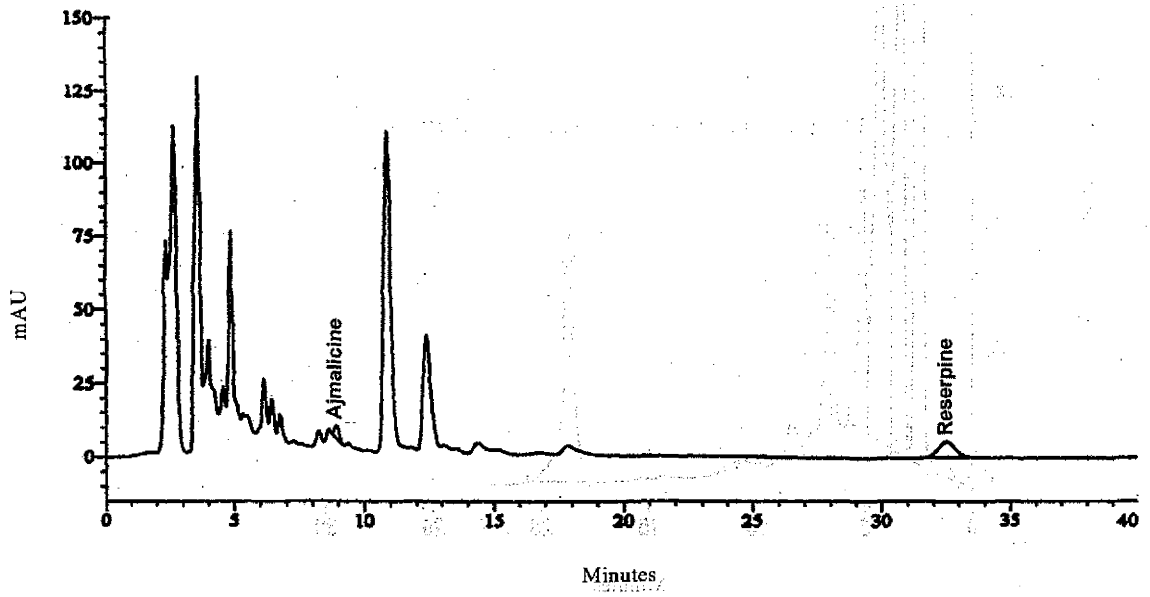


HPLC Chromatogram of Sarpagandha

Sarpagandha Powder

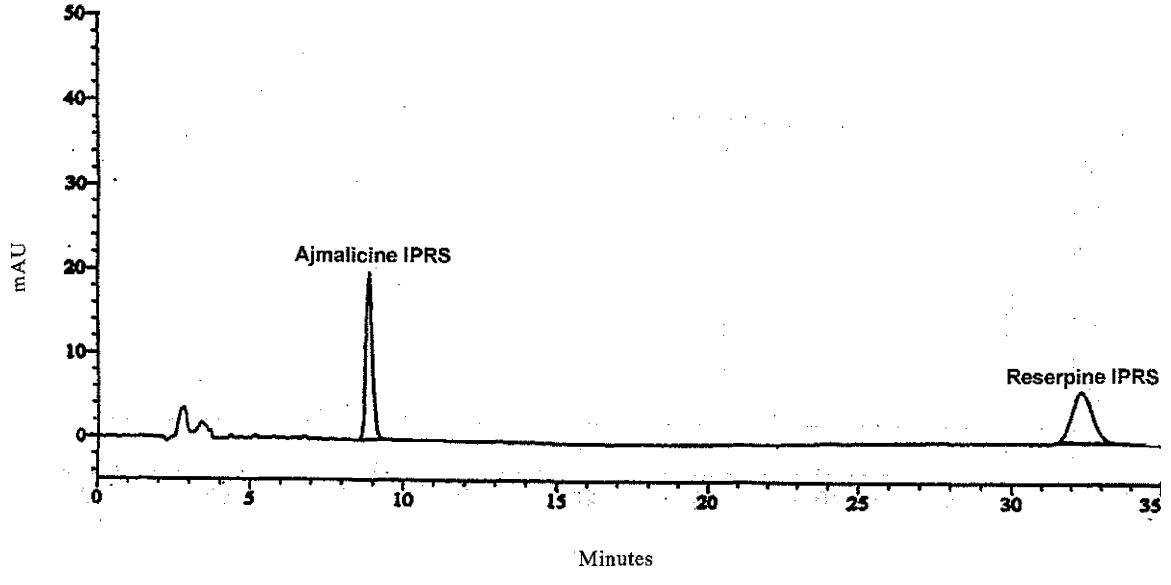


HPLC Chromatogram of ajmalicine IPRS and reserpine IPRS

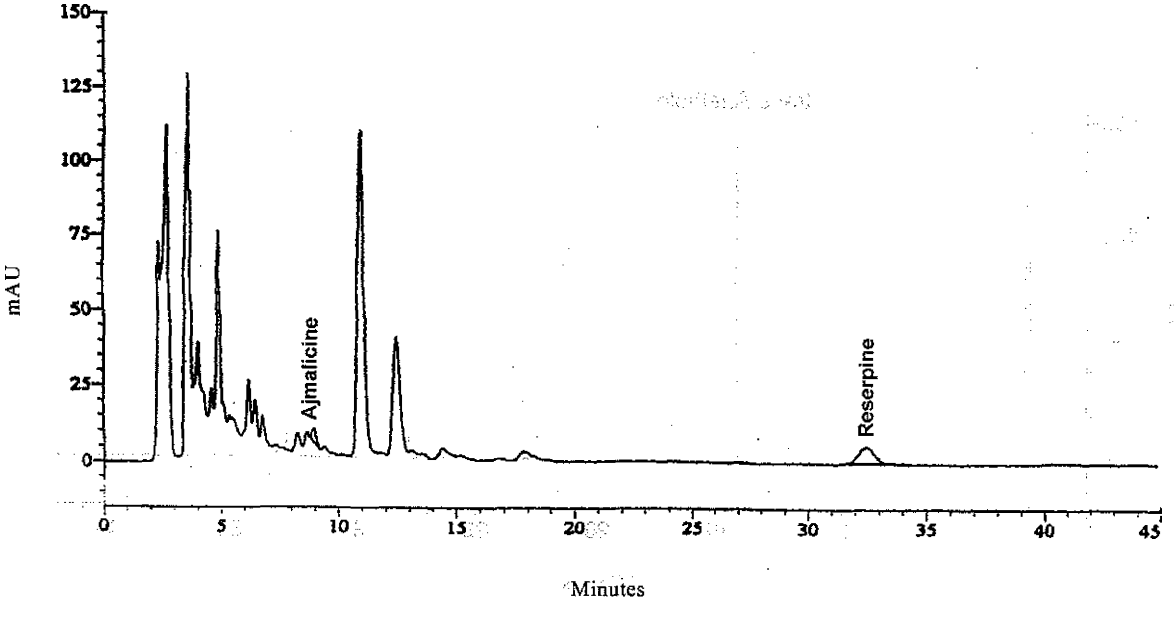


HPLC Chromatogram of Sarpagandha Powder

Sarpagandha Tablets

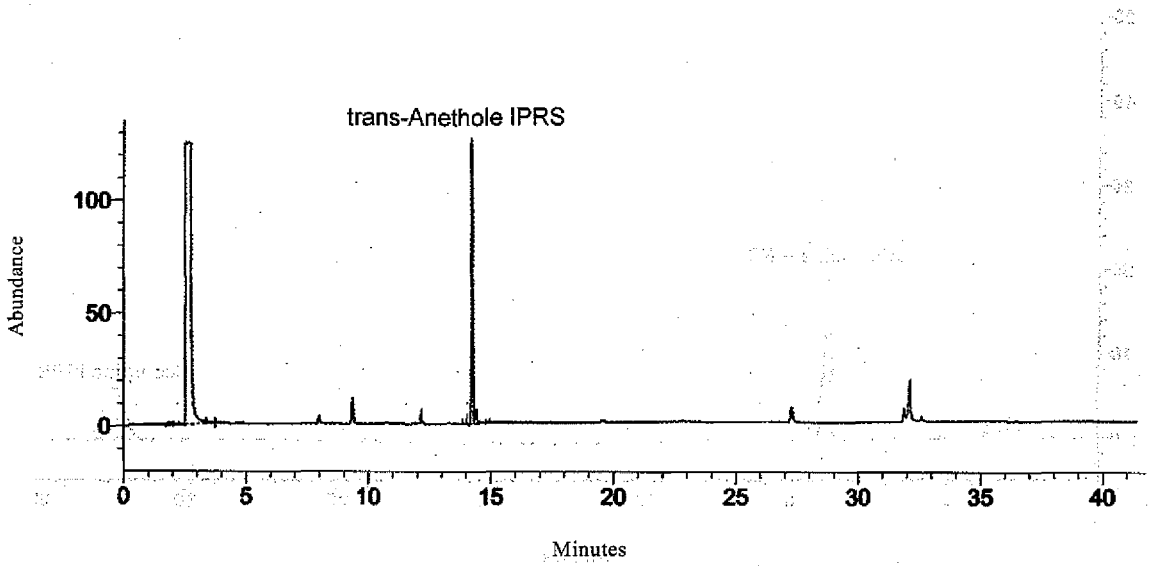


HPLC Chromatogram of ajmalicine IPRS and reserpine IPRS

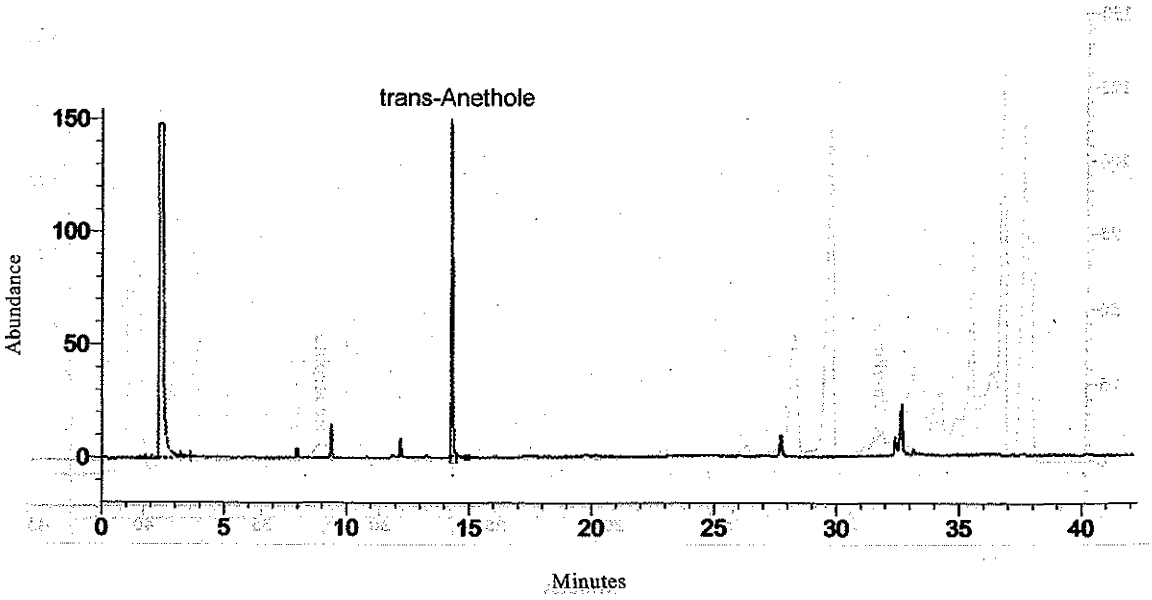


HPLC Chromatogram of Sarpagandha Tablets

Saunf

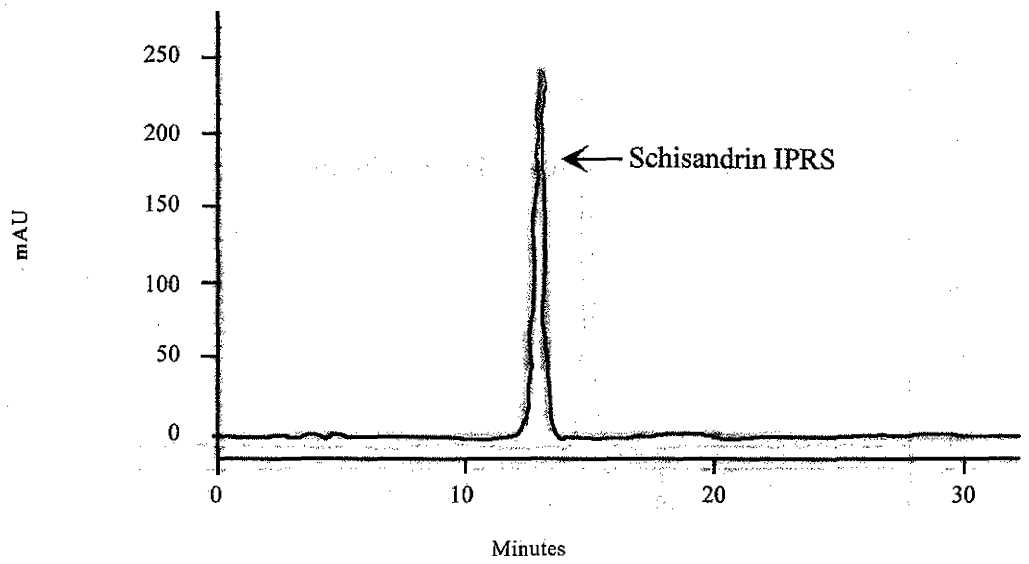


Gas Chromatogram of trans-anethole IPRS

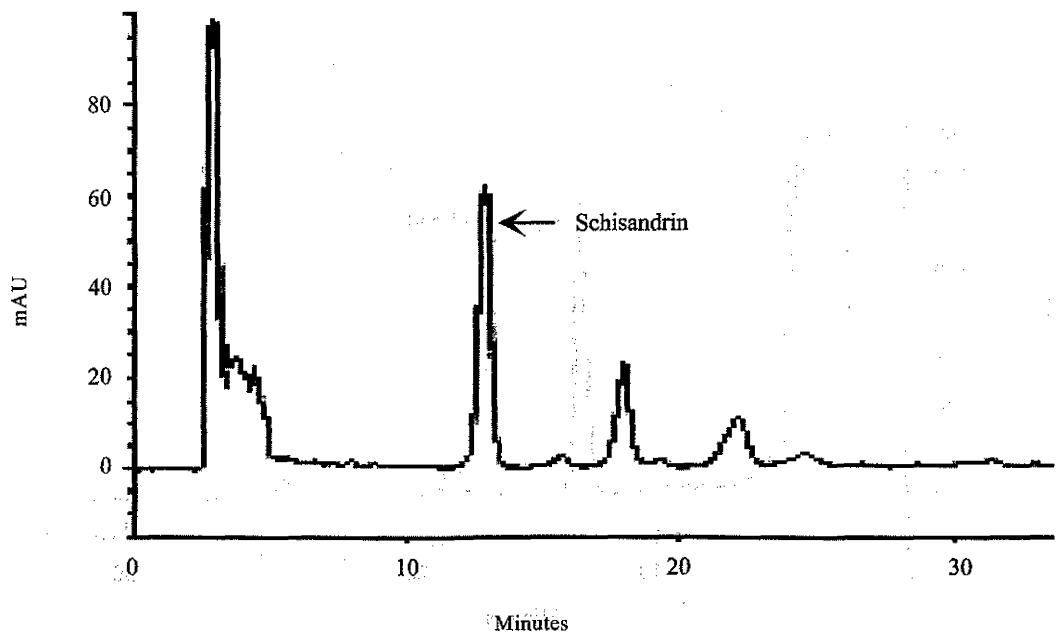


Gas Chromatogram of Saunf

Schisandra Fruit

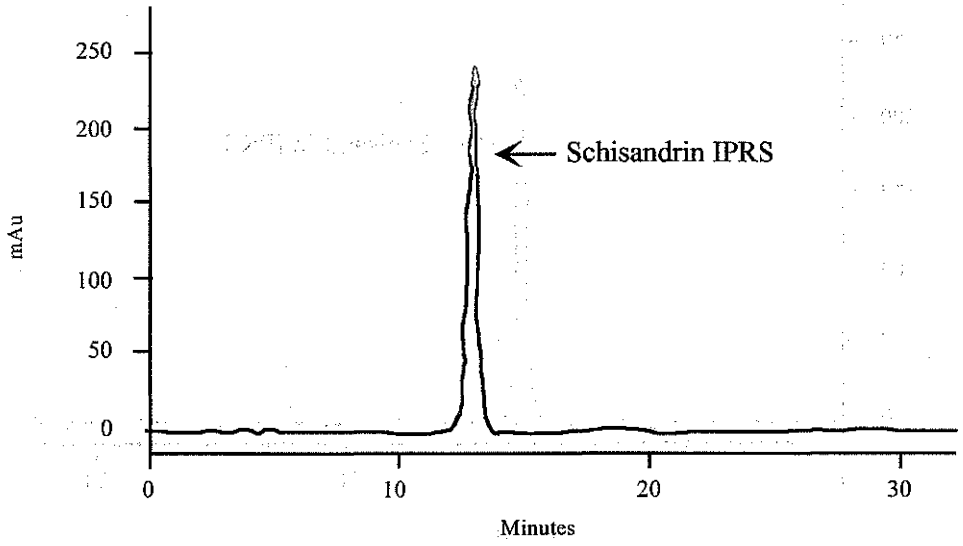


HPLC Chromatogram of schisandrin IPRS

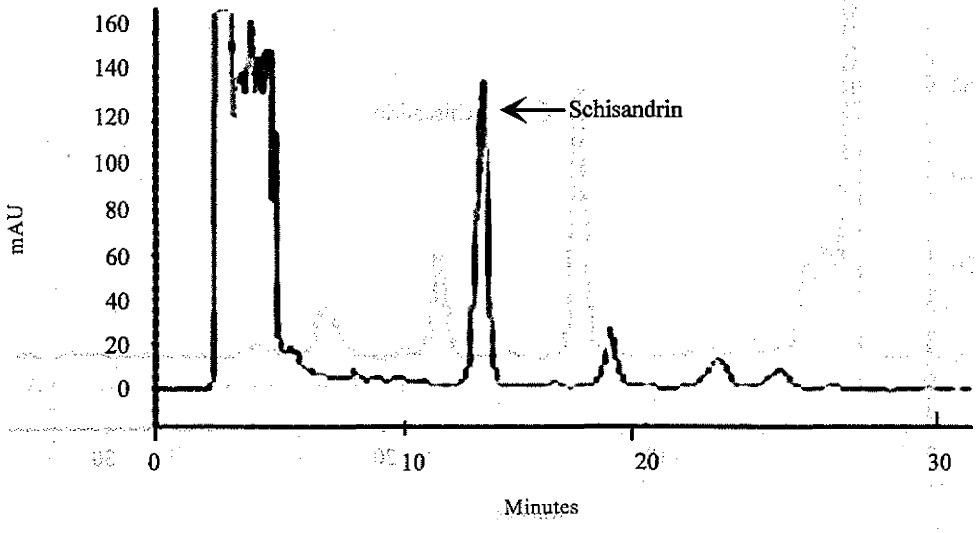


HPLC Chromatogram of Schisandra Fruit

Schisandra Dry Extract

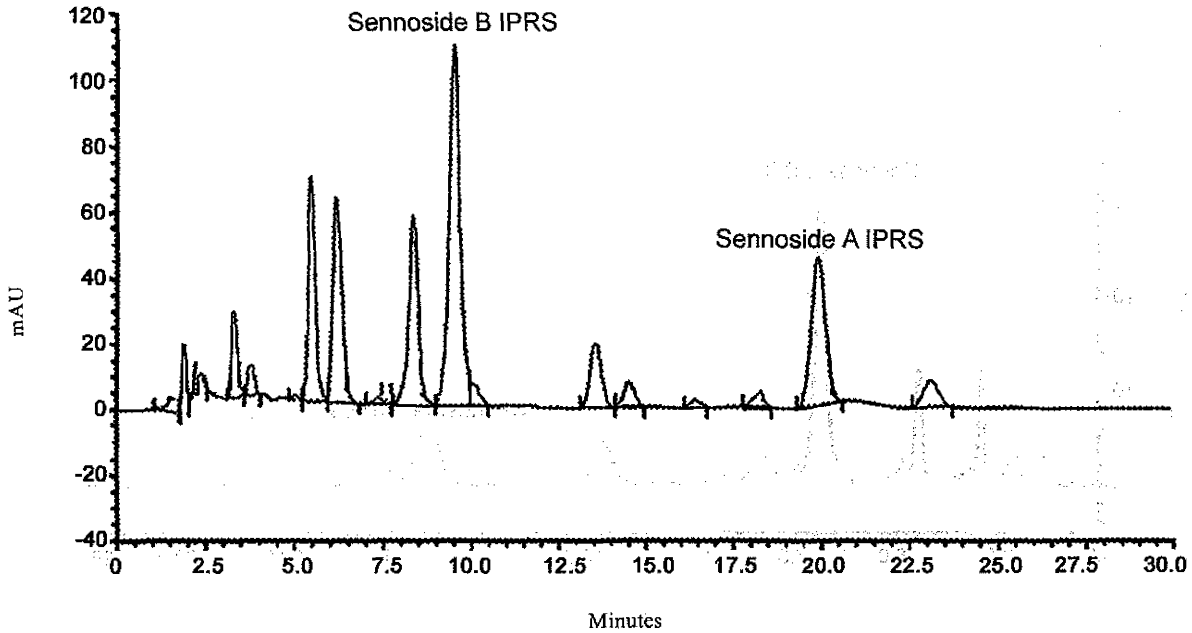


HPLC Chromatogram of schisandrin IPRS

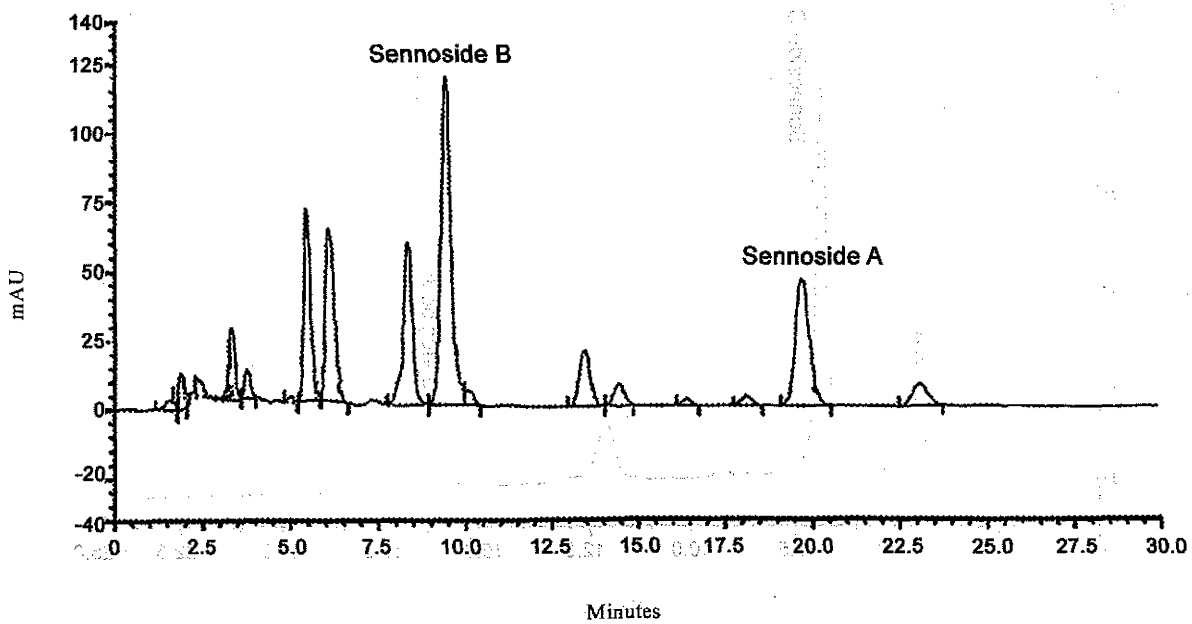


HPLC Chromatogram of Schisandra Dry Extract

Senna Pods

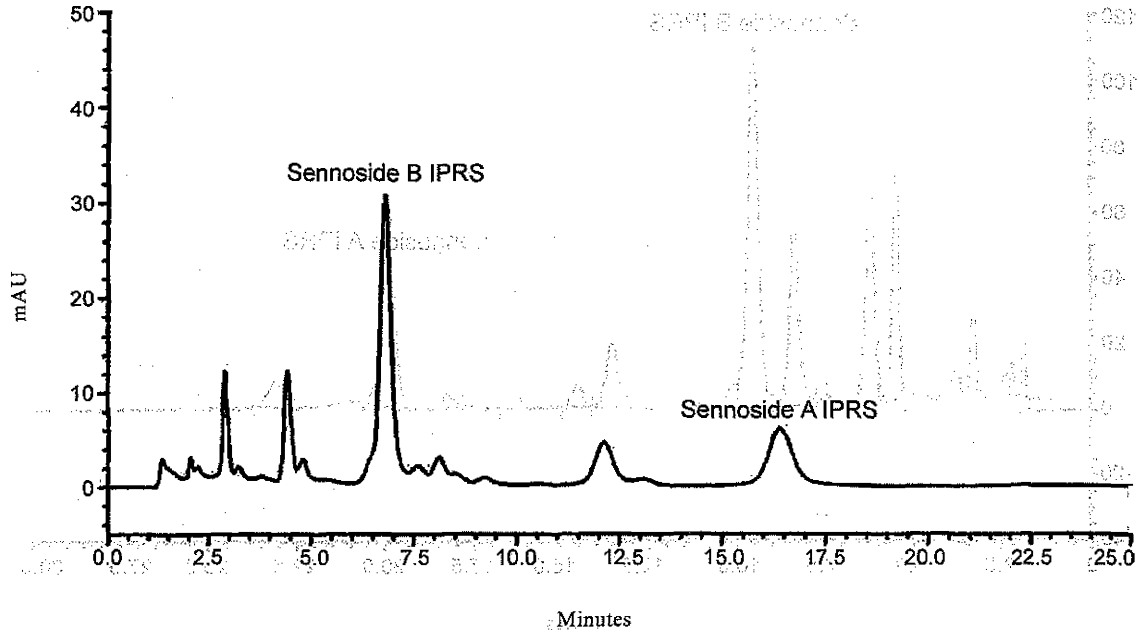


HPLC Chromatogram of sennoside A IPRS and sennoside B IPRS

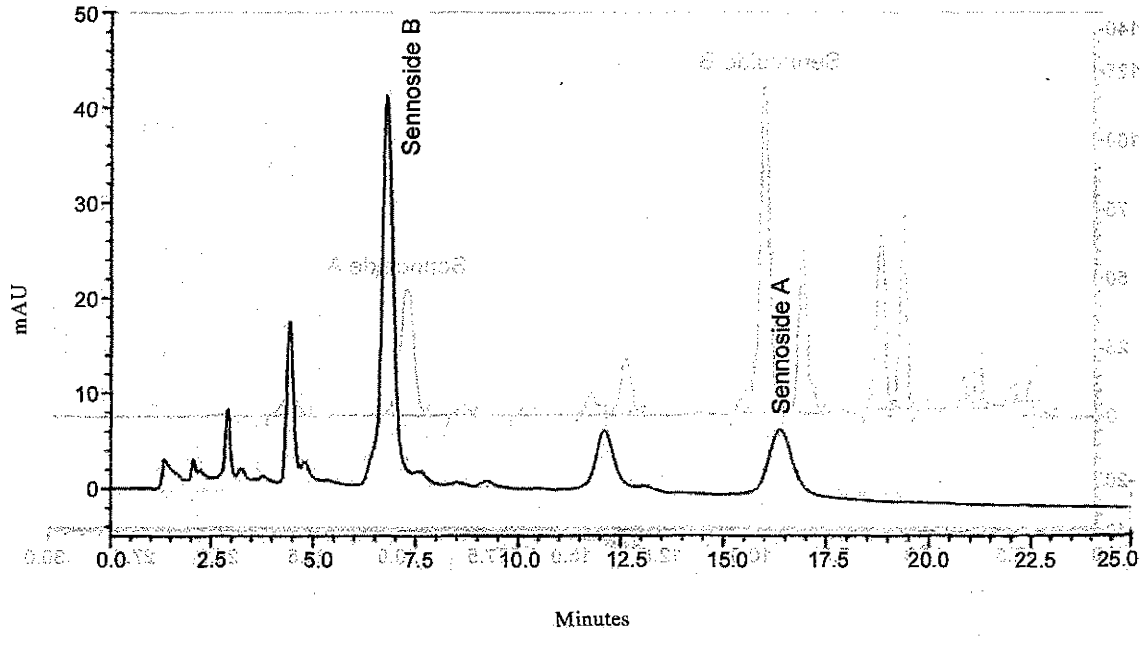


HPLC Chromatogram of Senna Pods

Senna Dry Extract

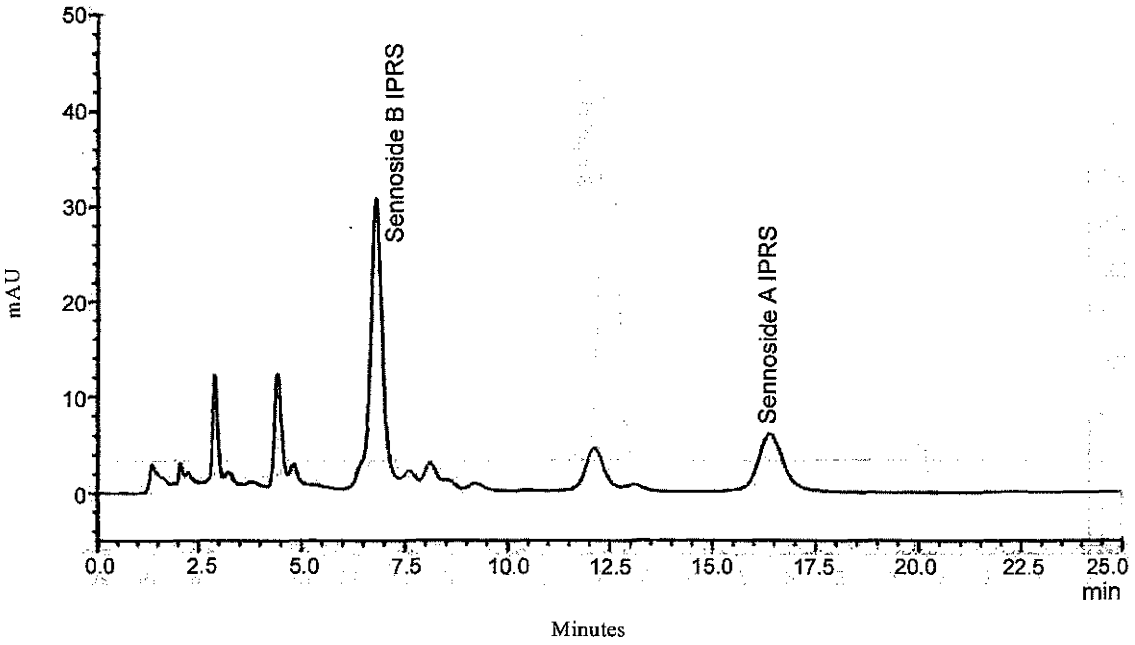


HPLC Chromatogram of sennoside A IPRS and sennoside B IPRS

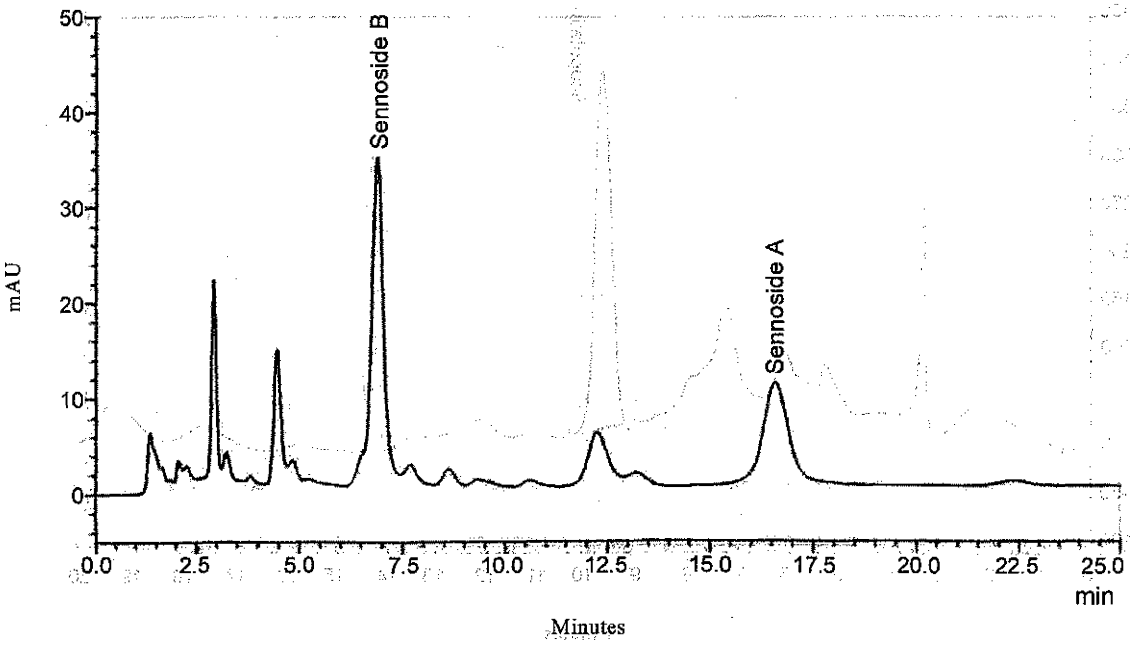


HPLC Chromatogram of Senna Dry Extract

Senna Tablets

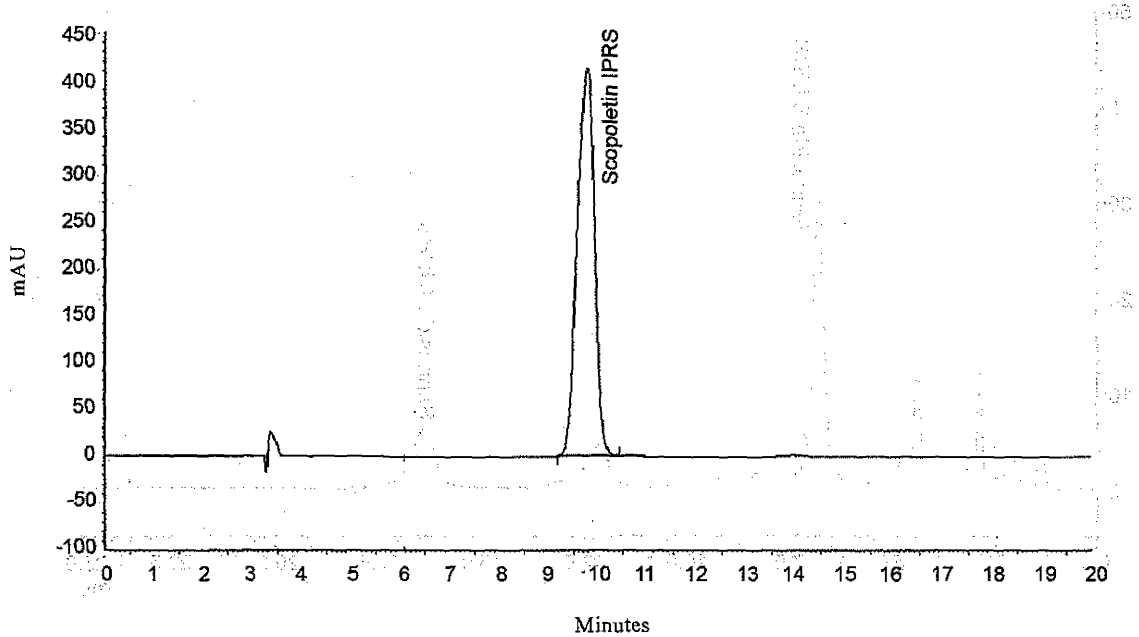


HPLC Chromatogram of sennoside A IPRS and sennoside B IPRS

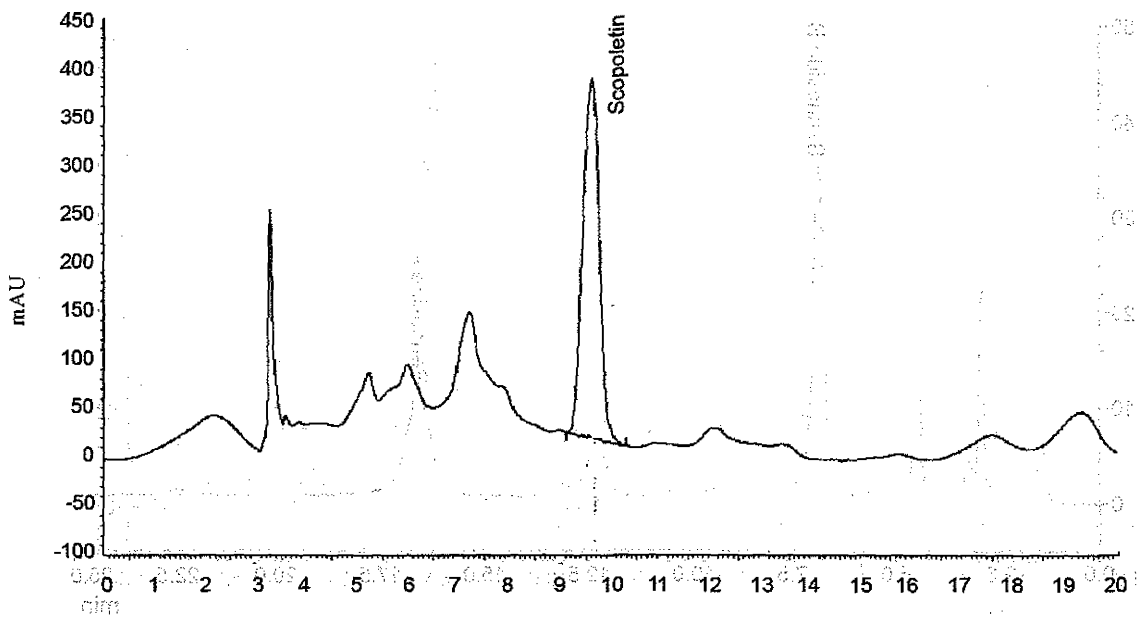


HPLC Chromatogram of Senna Tablets

Shankhpushpi

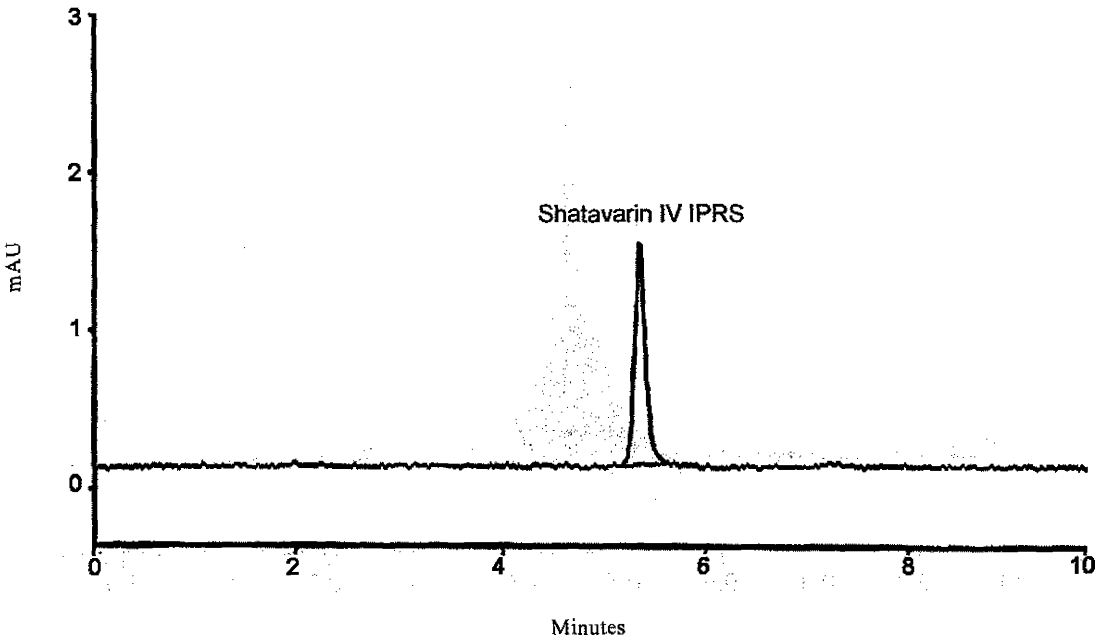


HPLC Chromatogram of scopoletin IPRS

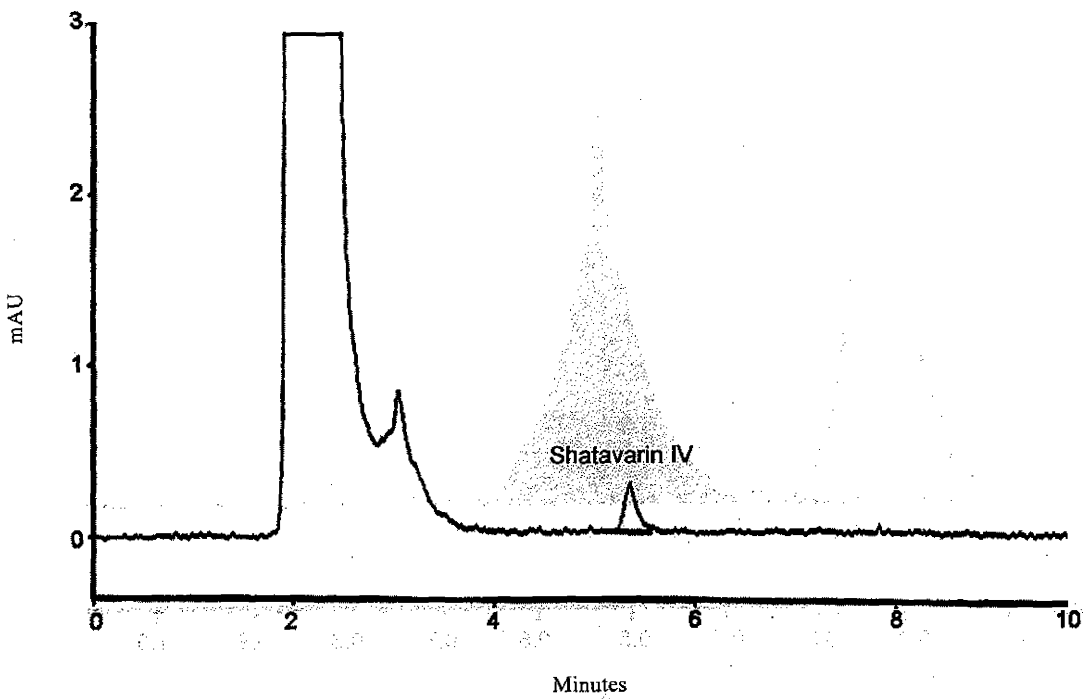


HPLC Chromatogram of Shankhpushpi

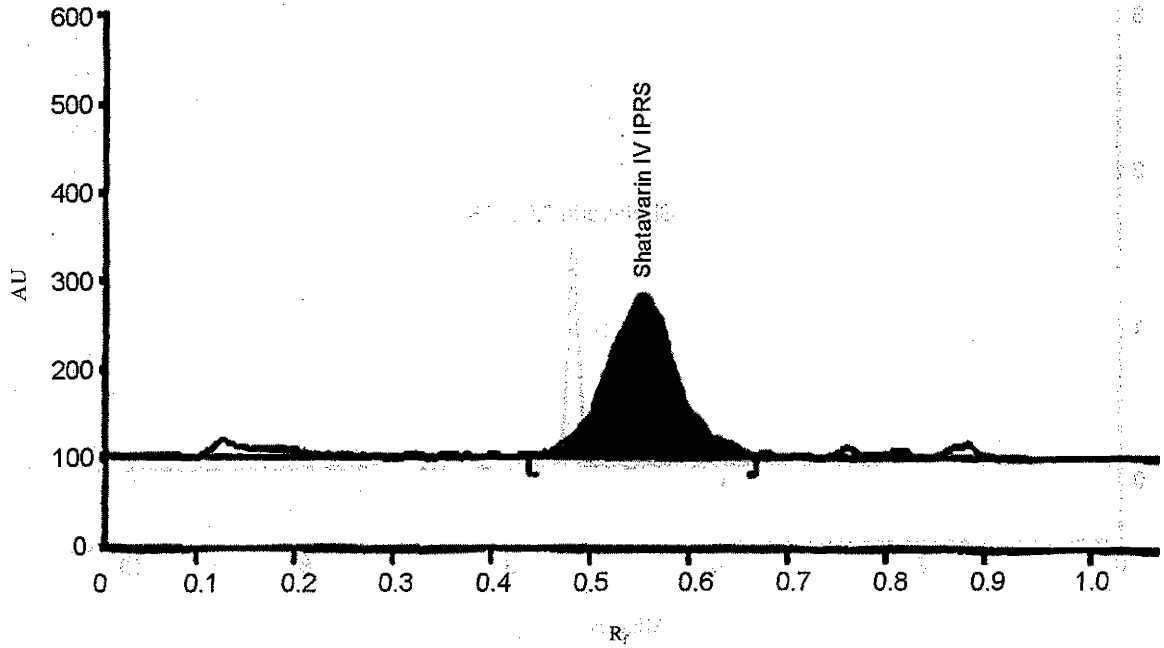
Shatavari



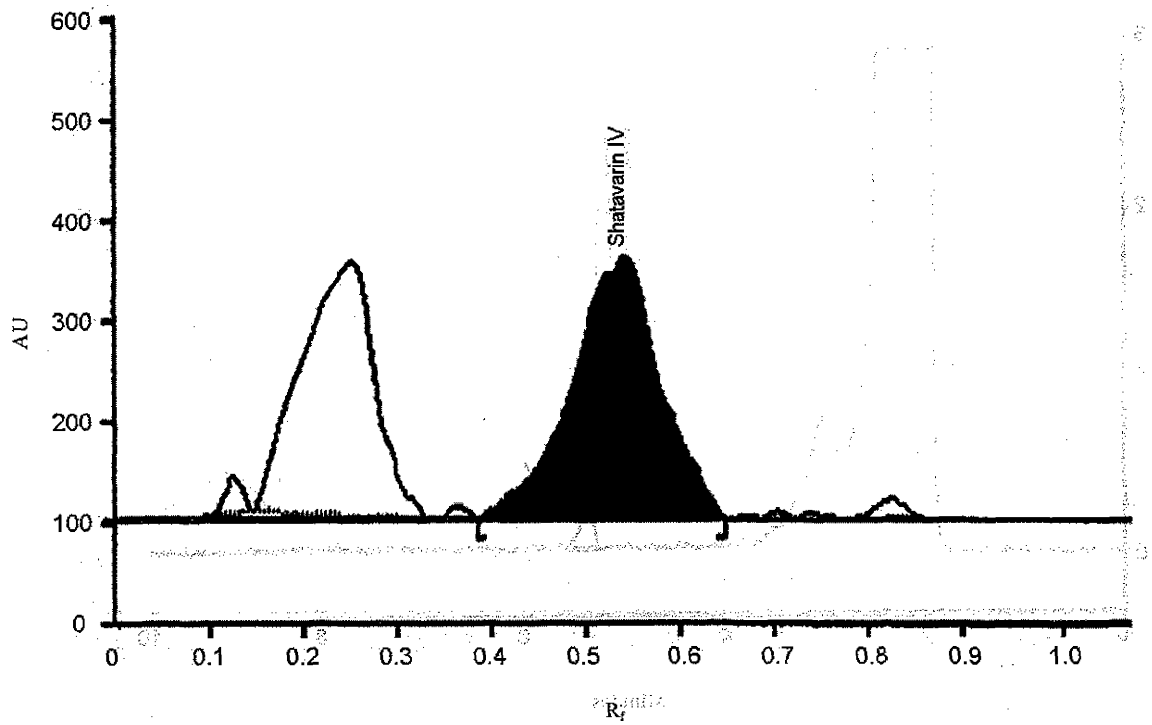
HPLC Chromatogram of shatavarin IV IPRS



HPLC Chromatogram of Shatavari

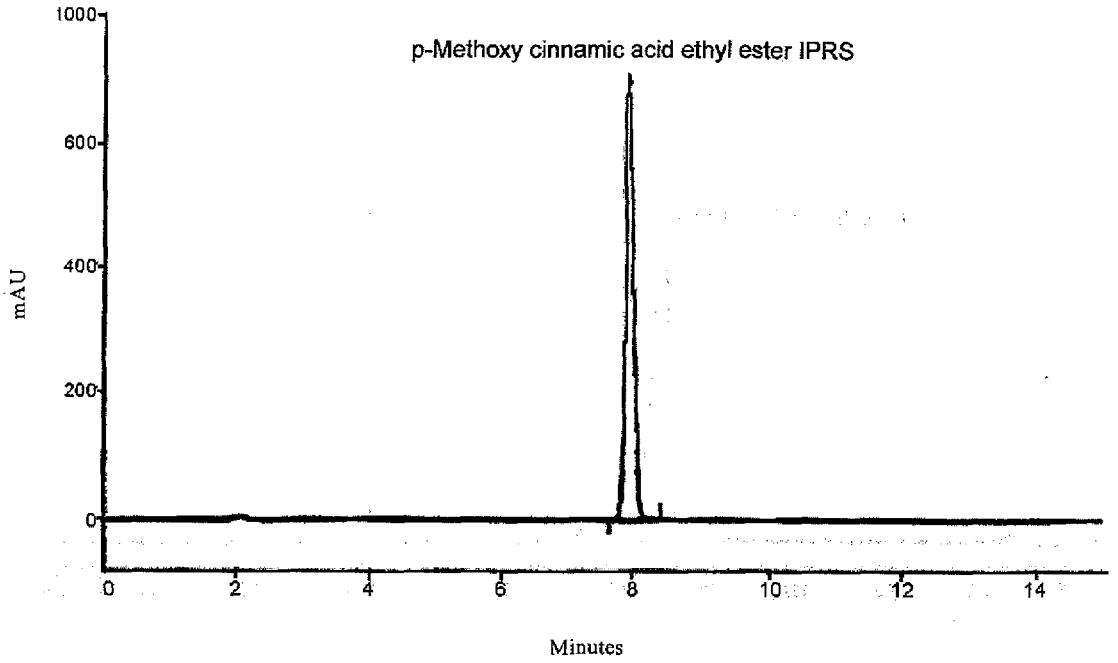


HPTLC Chromatogram of shatavarin IV IPRS

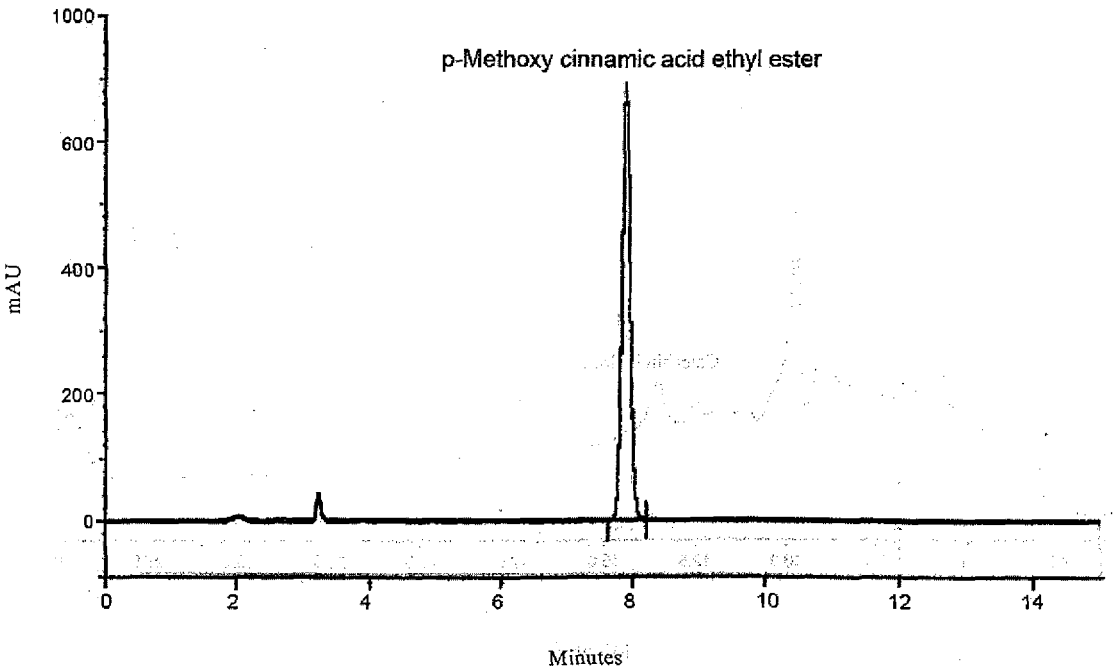


HPTLC Chromatogram of Shatavari

Shati

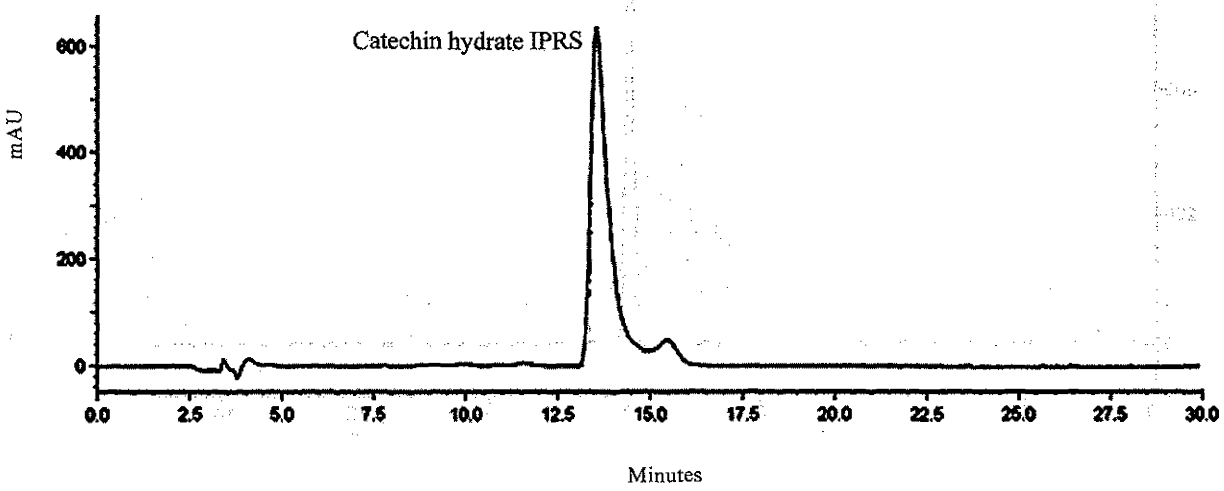


HPLC Chromatogram of p-methoxy cinnamic acid ethyl ester IPRS

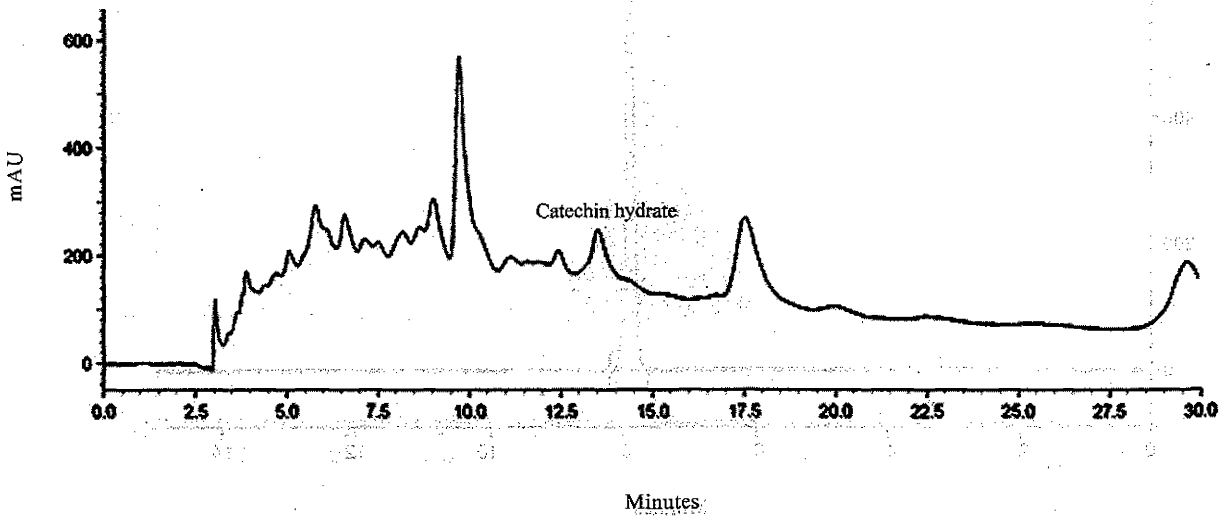


HPLC Chromatogram of Shati

Siris

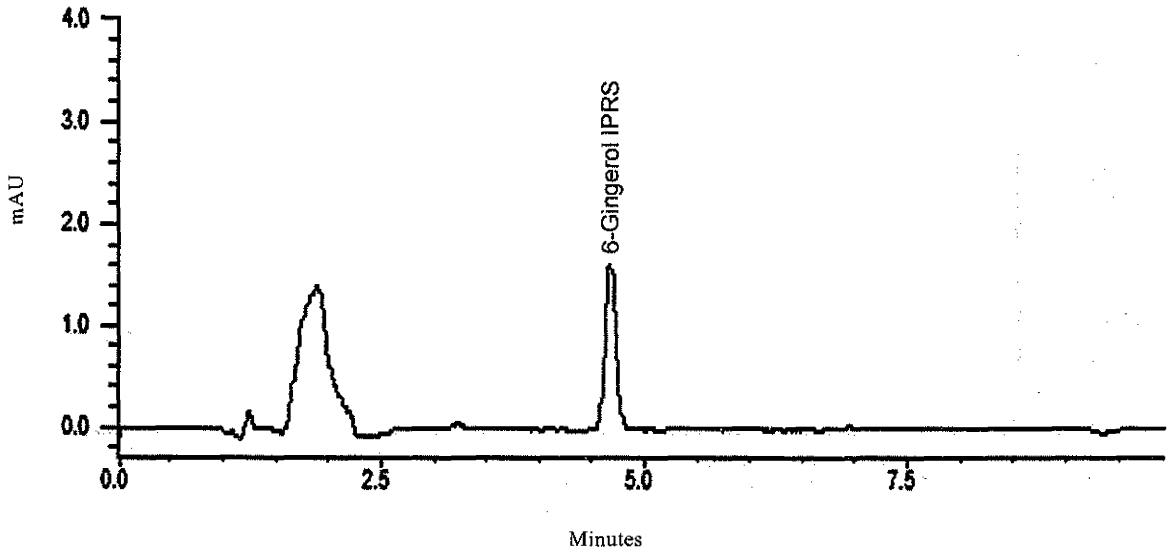


HPLC Chromatogram of catechin hydrate IPRS

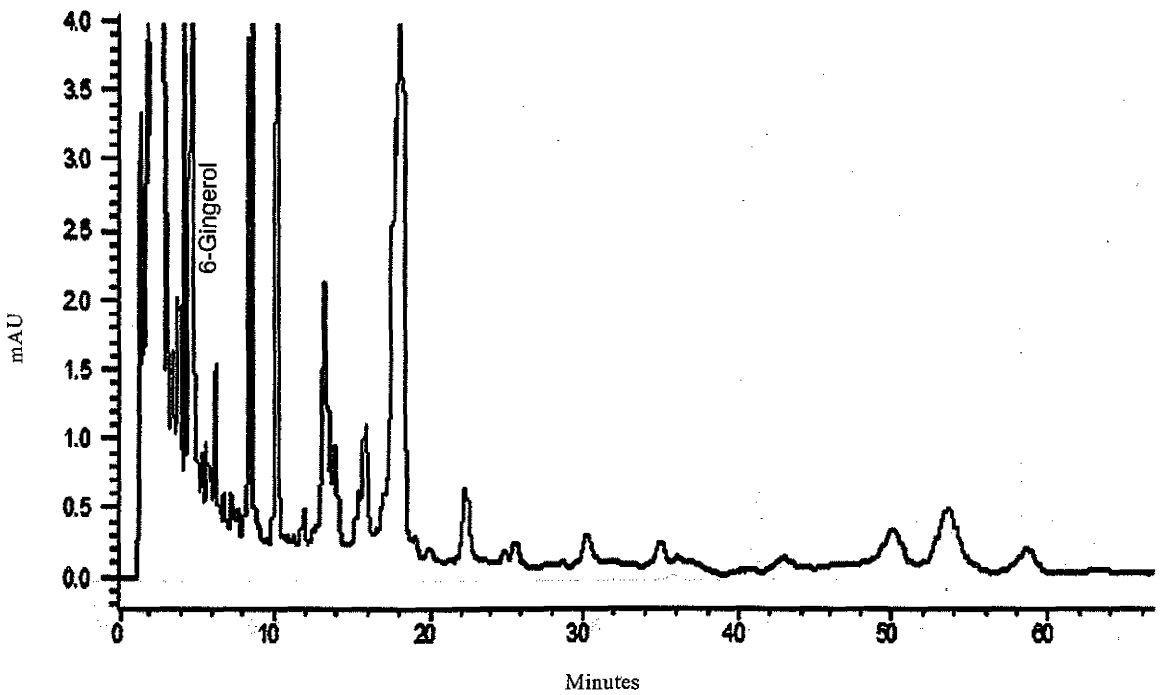


HPLC Chromatogram of Siris

Sunthi

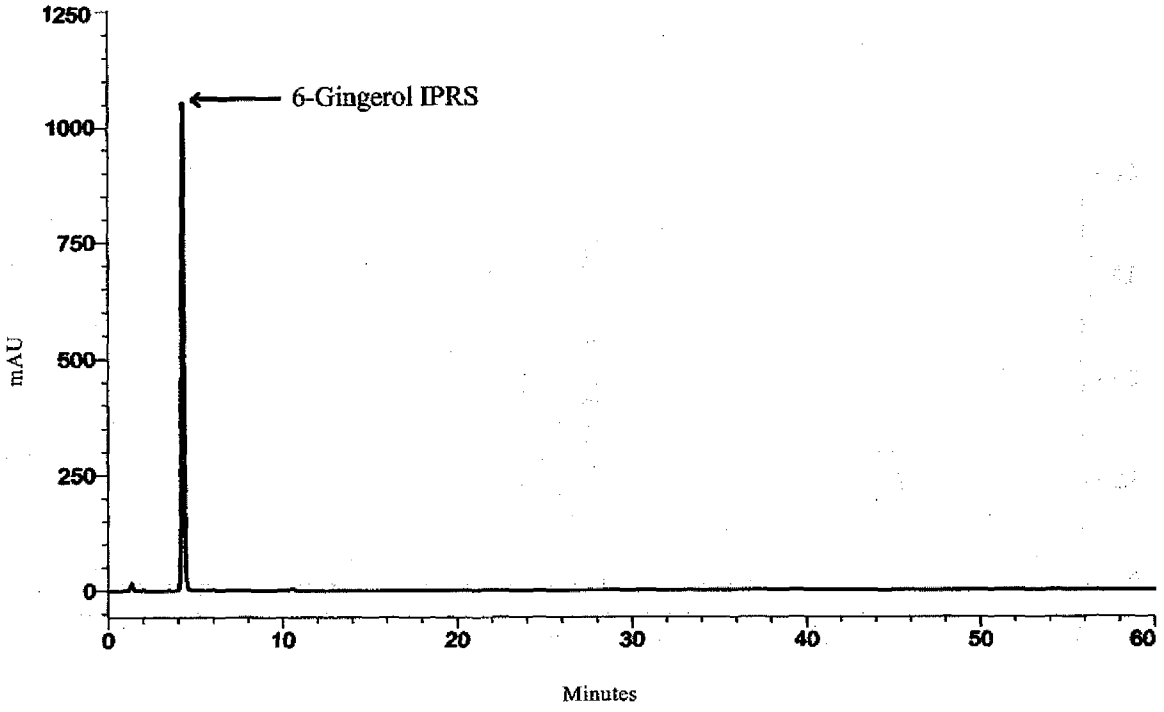


HPLC Chromatogram of 6-gingerol IPRS

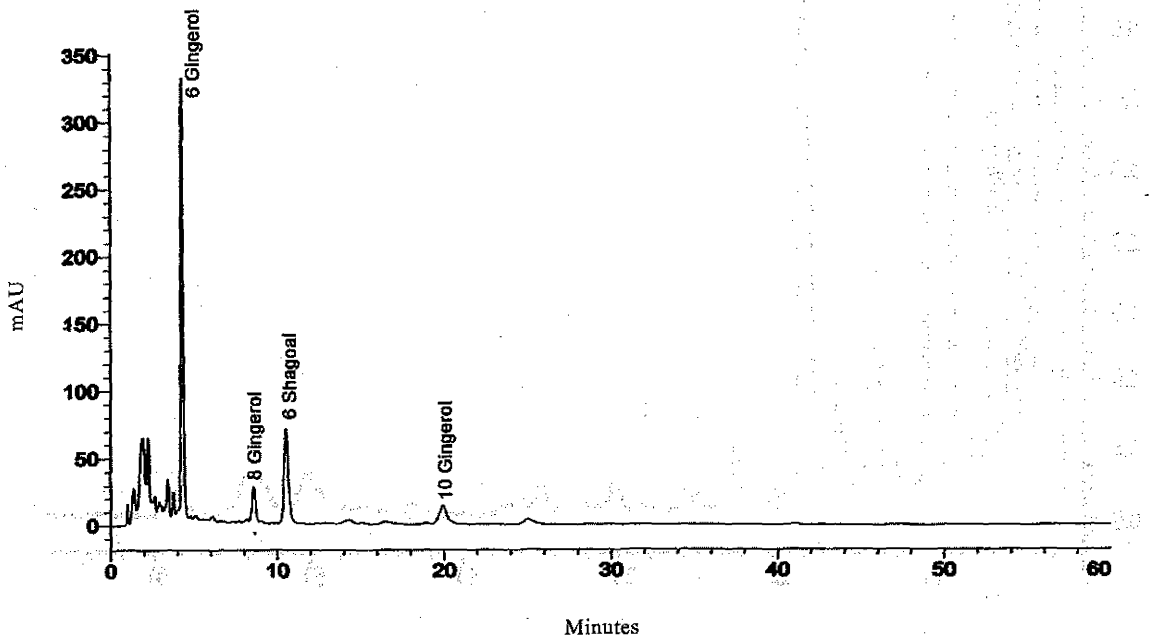


HPLC Chromatogram of Sunthi

Sunthi Extract

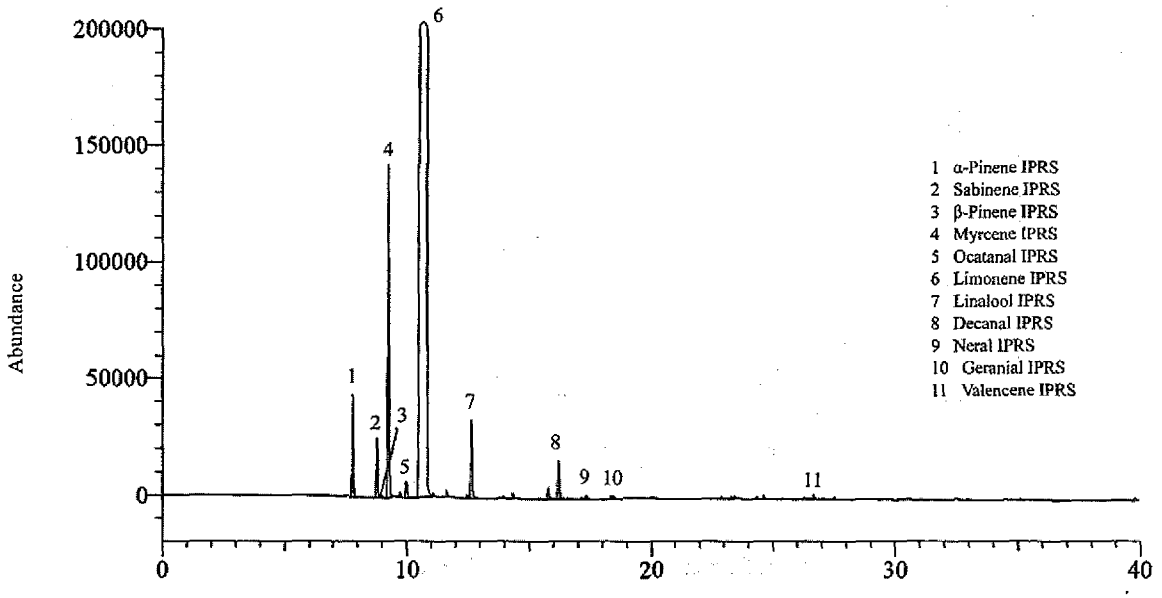


HPLC Chromatogram of 6-gingerol IPRS



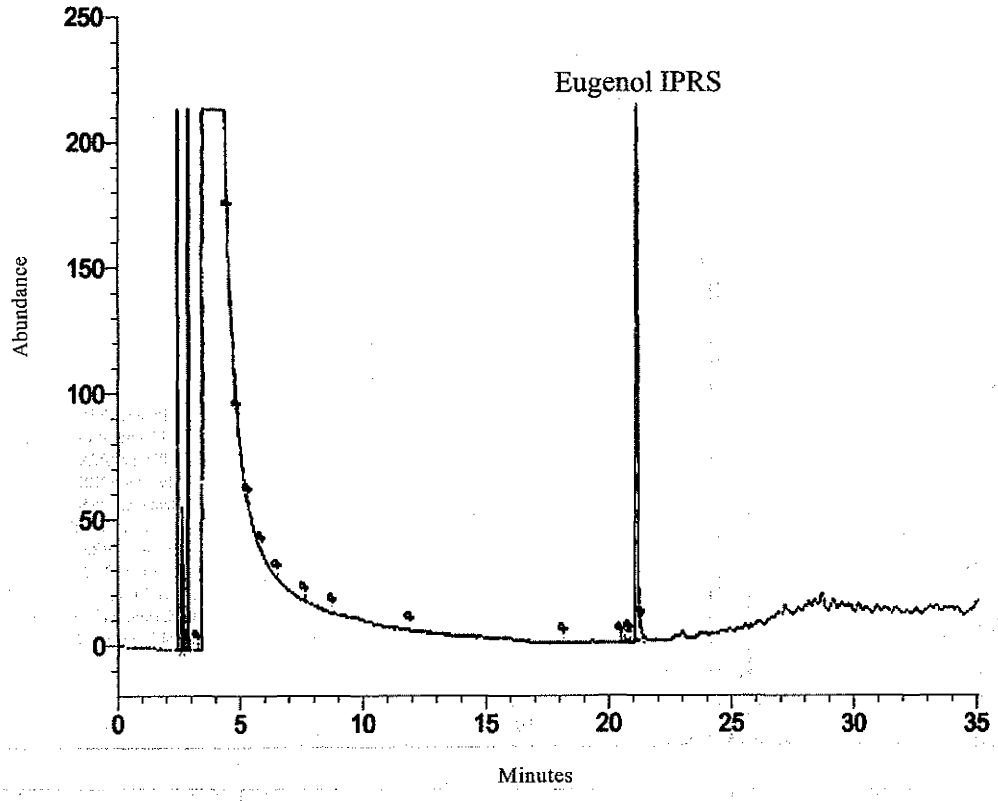
HPLC Chromatogram of Sunthi Extract

Sweet Orange Oil

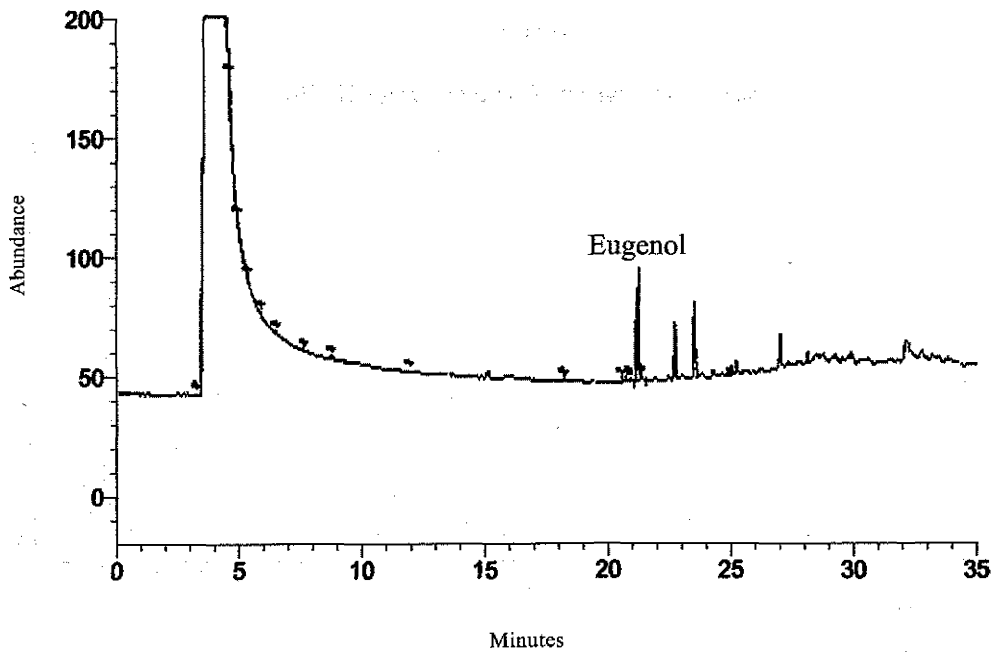


Gas Chromatogram of Sweet Orange Oil IPRS

Tulasi

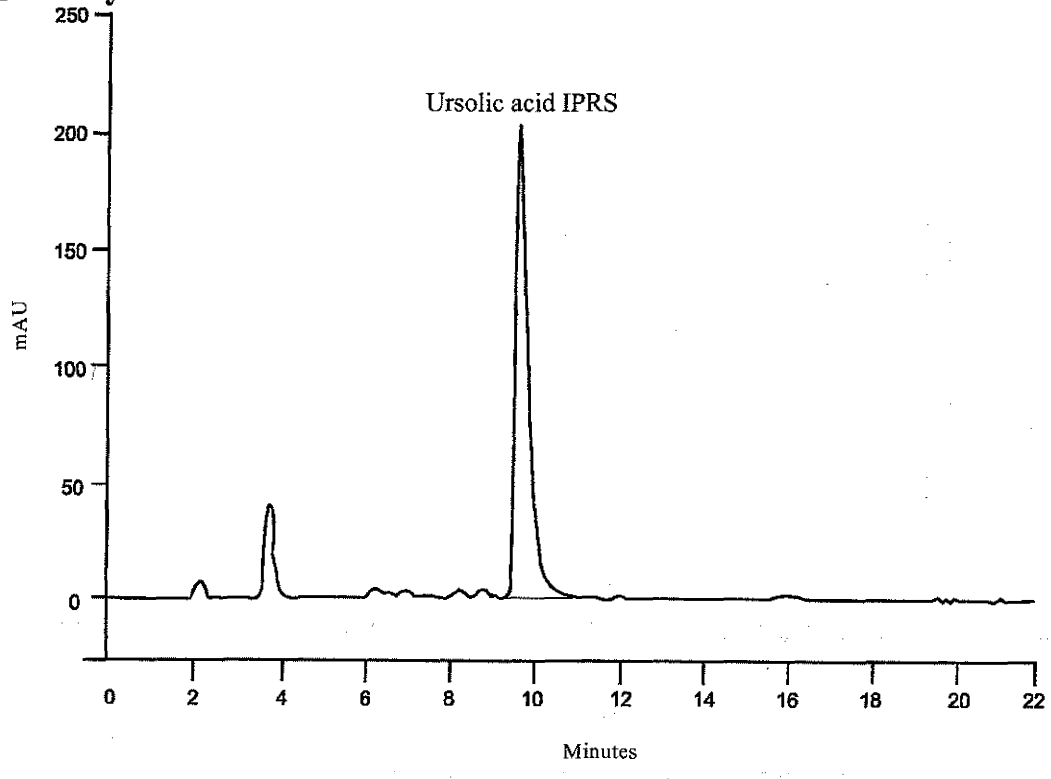


Gas Chromatogram of eugenol IPRS

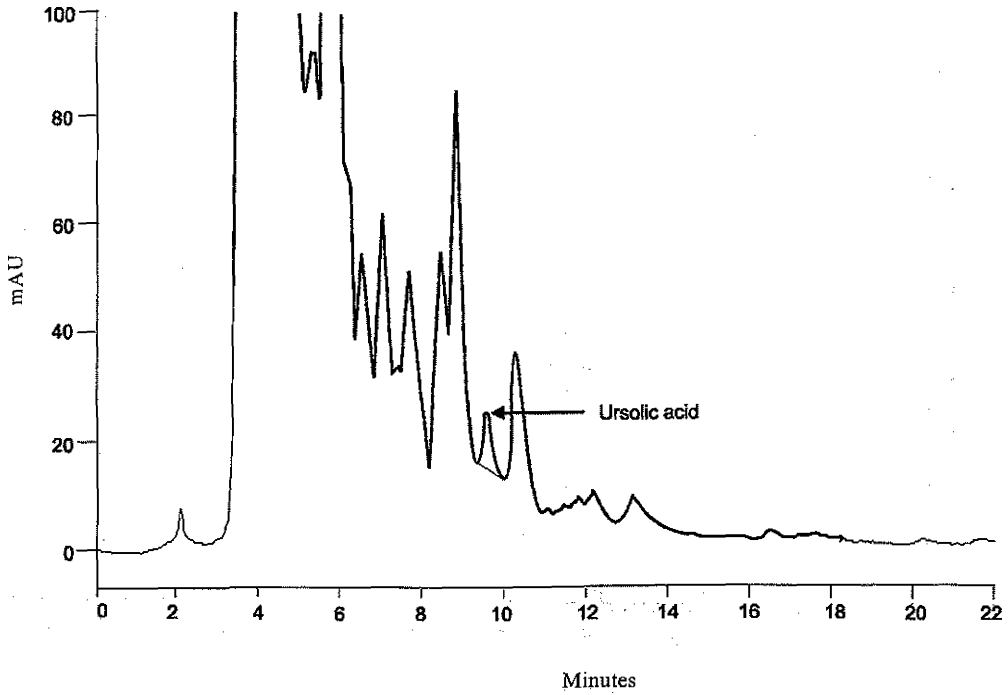


Gas Chromatogram of Tulasi

Tulasi Dry Extract

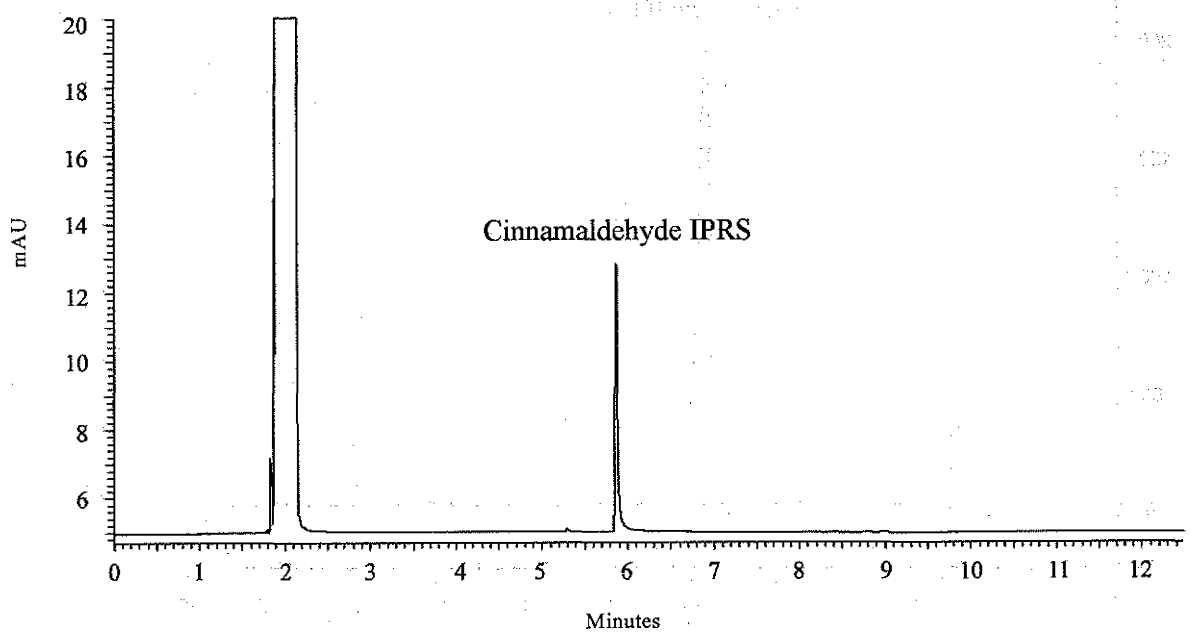


HPLC Chromatogram of ursolic acid IPRS

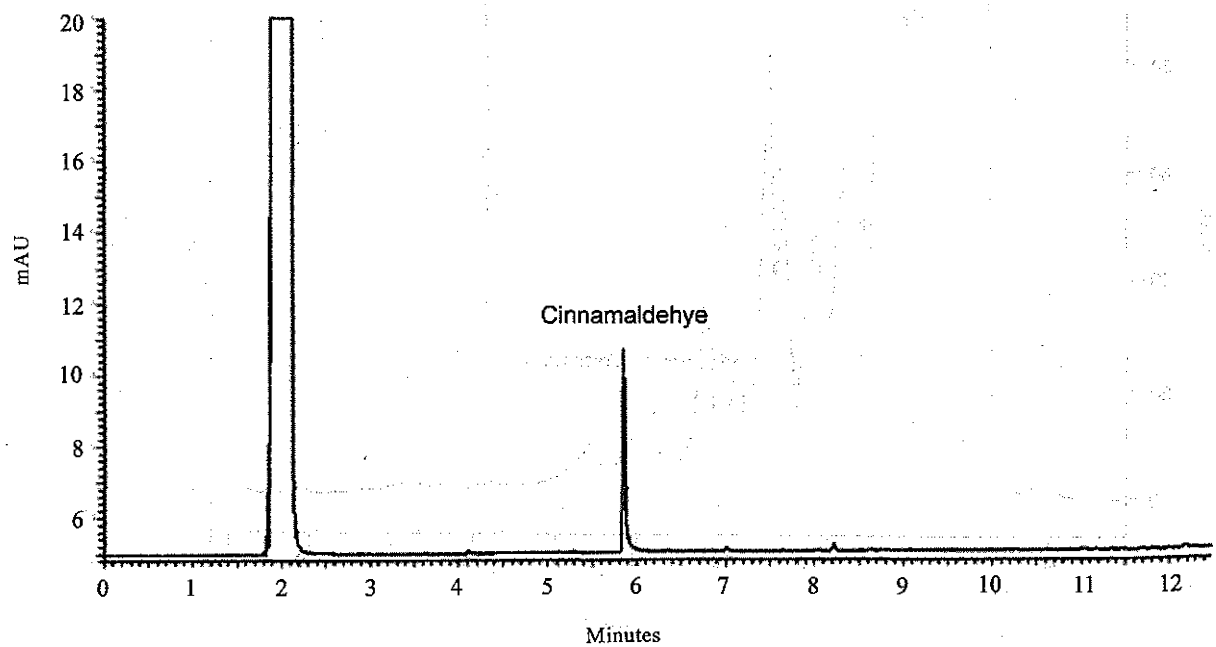


HPLC Chromatogram of Tulasi Dry Extract

Tvak

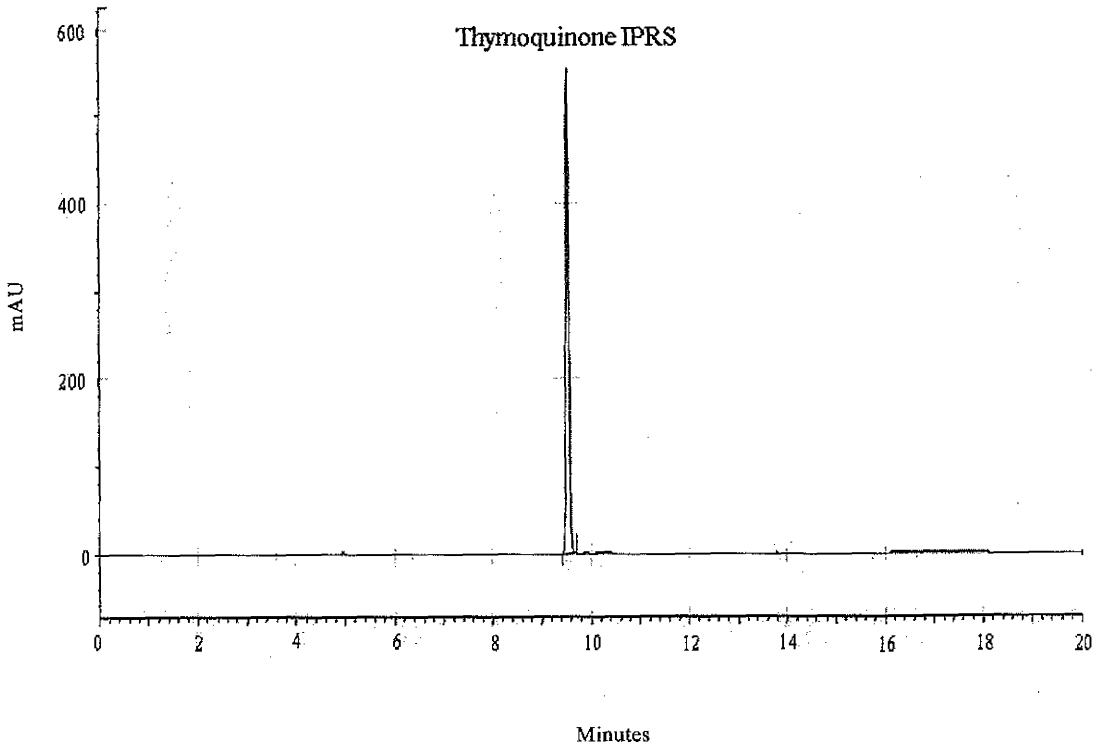


HPLC Chromatogram of cinnamaldehyde IPRS

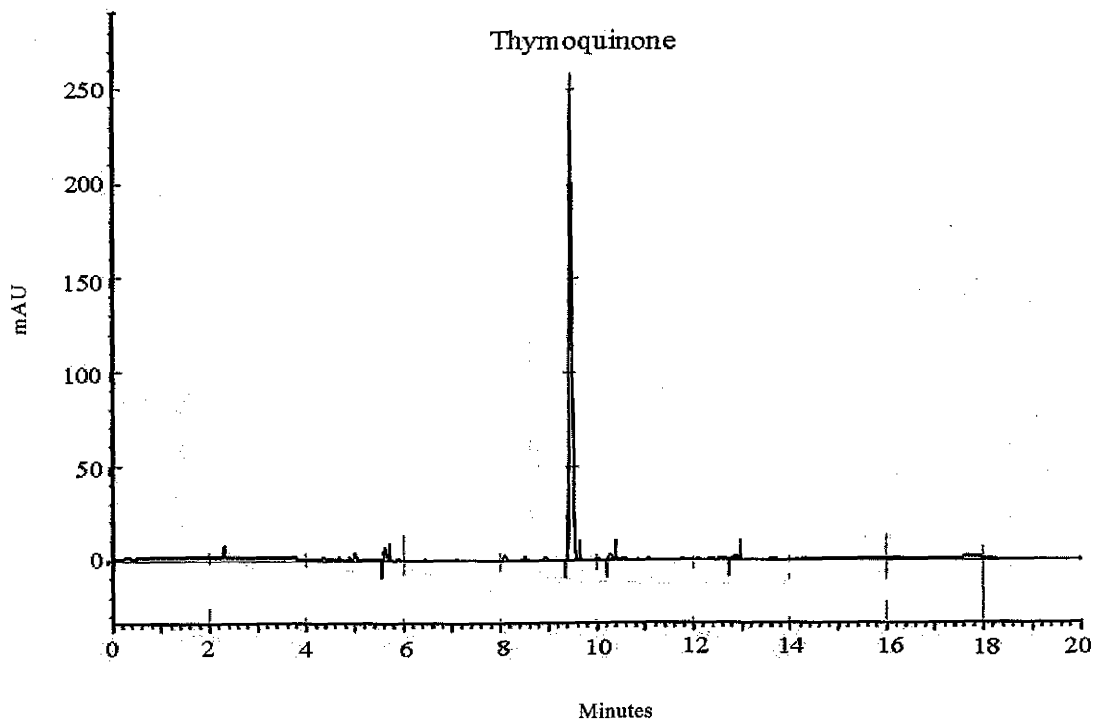


HPLC Chromatogram of Tvak

Upakunchika

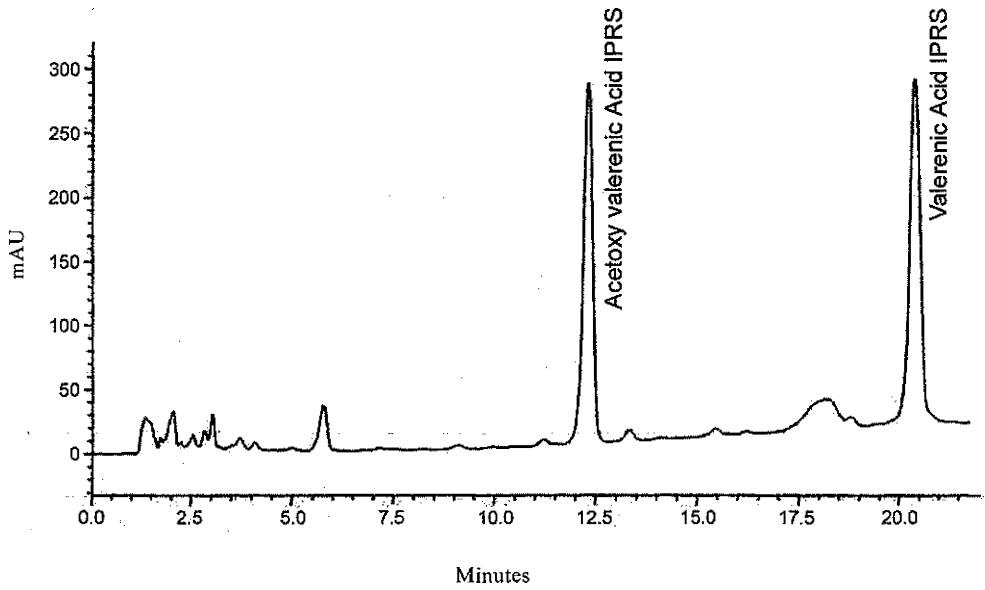


HPLC Chromatogram of thymoquinone IPRS

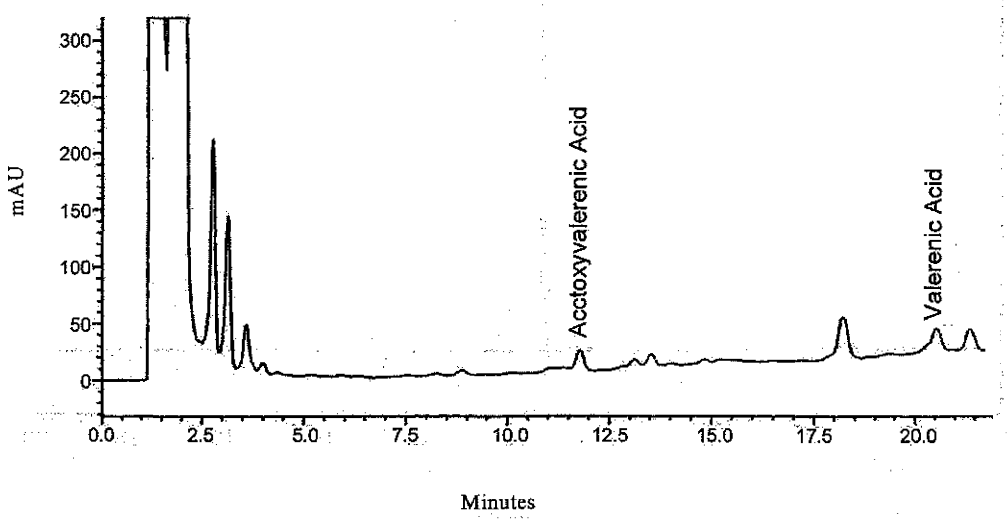


HPLC Chromatogram of Upakunchika

Valerian Root

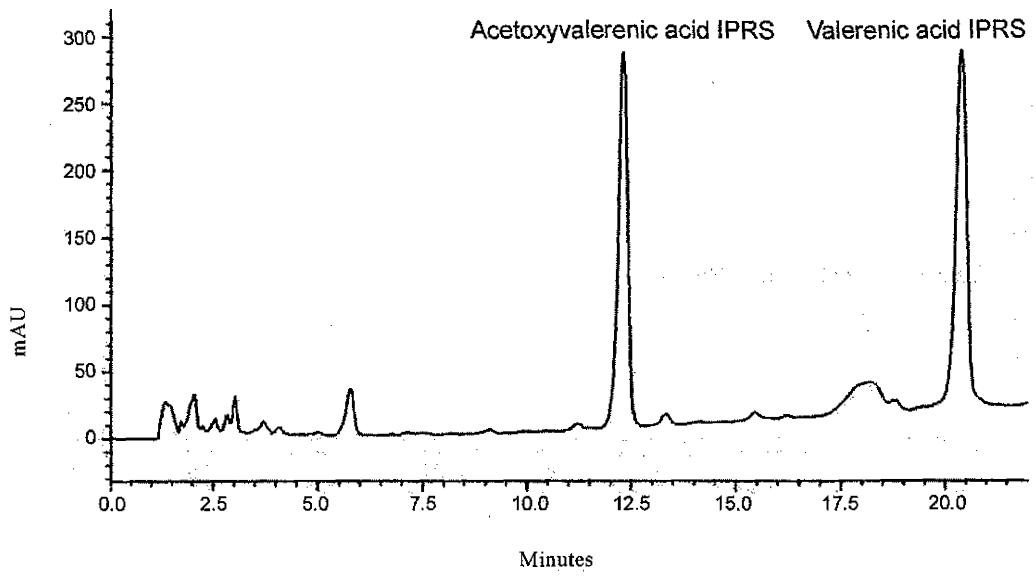


HPLC Chromatogram of valerenic acid IPRS

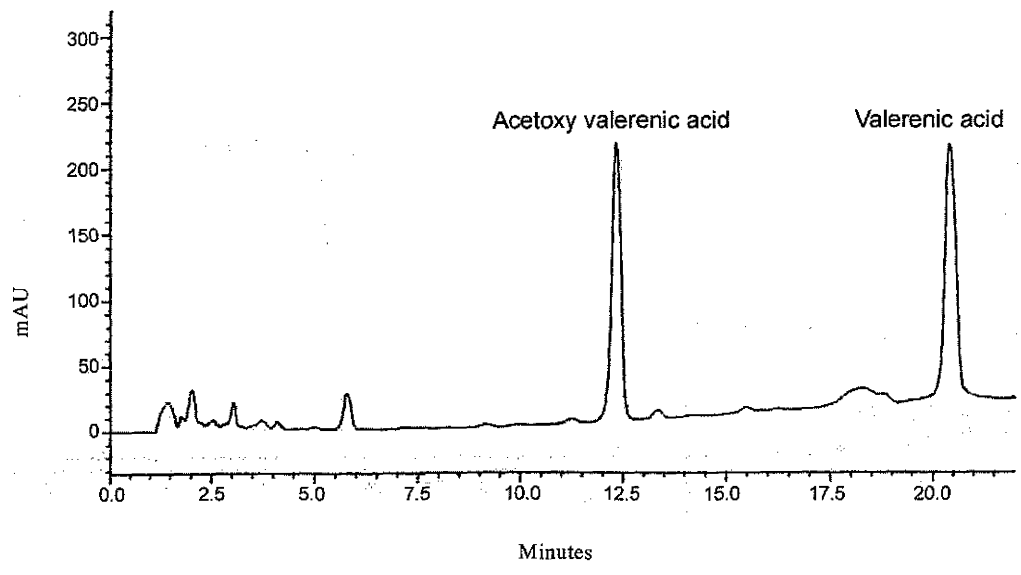


HPLC Chromatogram of Valerian Root

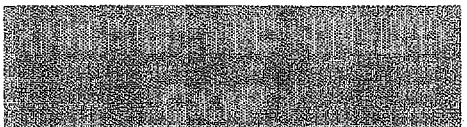
Valerian Dry Extract



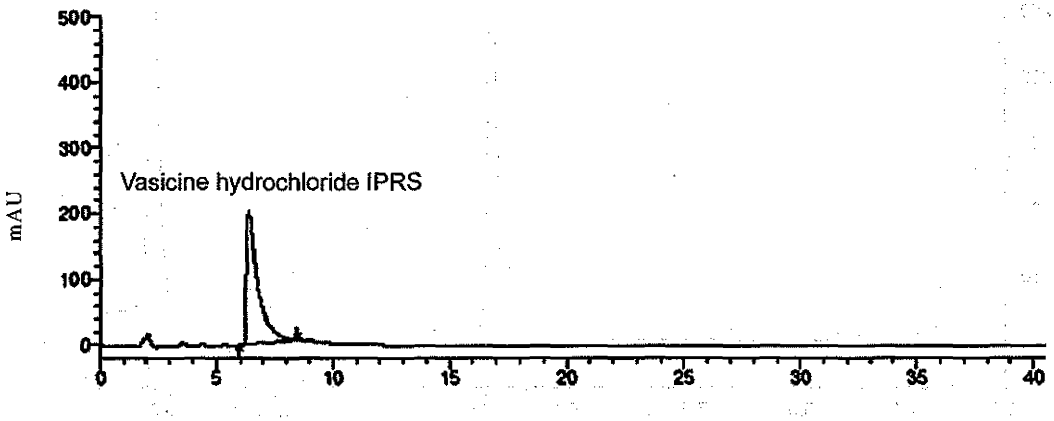
HPLC Chromatogram of valerenic acid IPRS



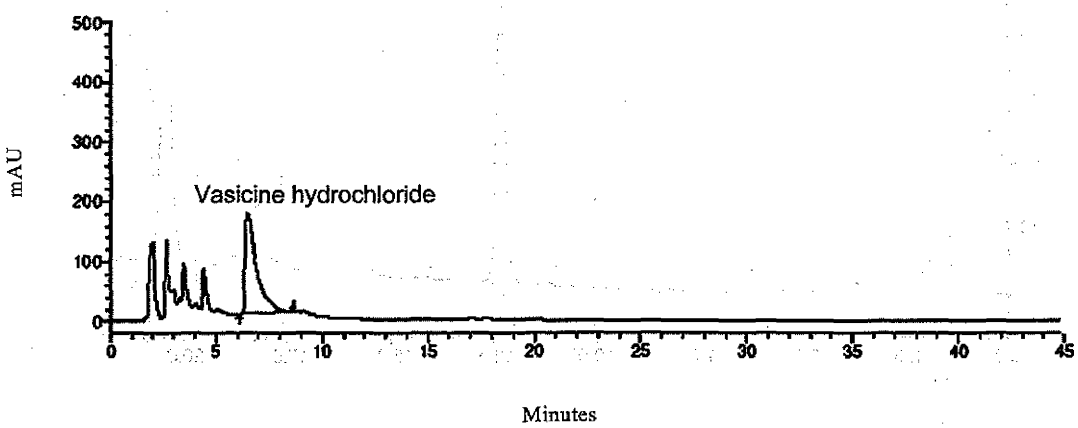
HPLC Chromatogram of Valerian Dry Extract



Vasaka

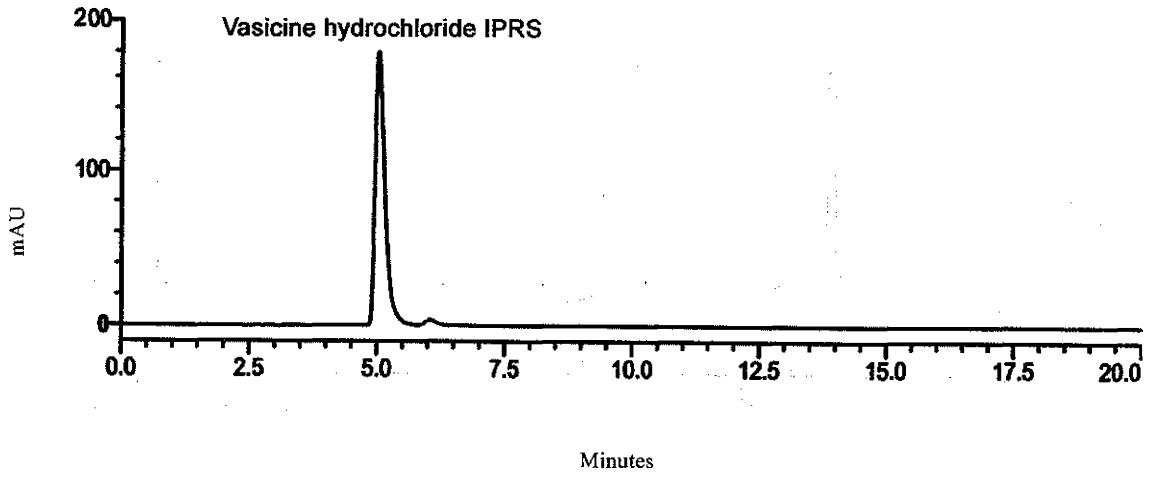


HPLC Chromatogram of vasicine hydrochloride IPRS

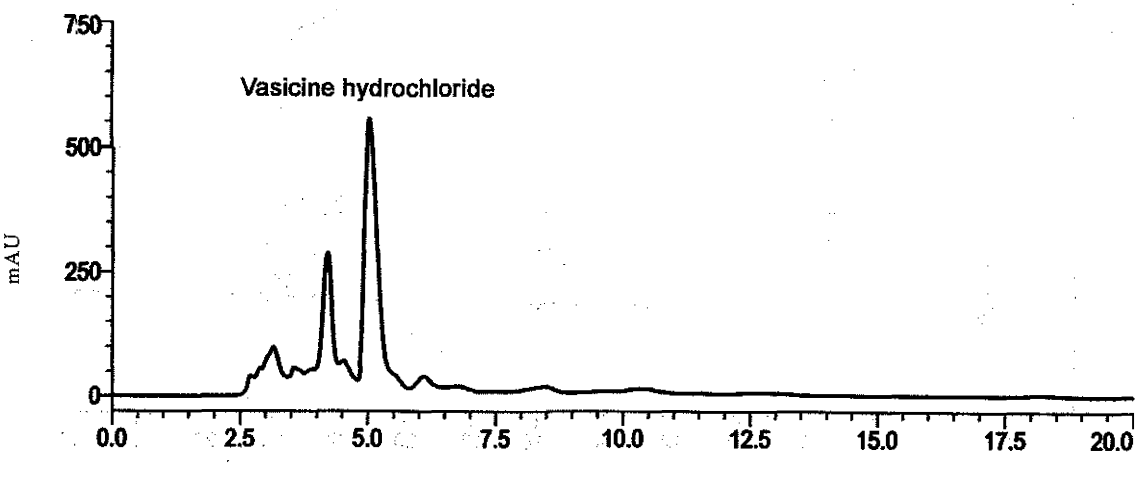


HPLC Chromatogram of Vasaka

Vasaka Extract

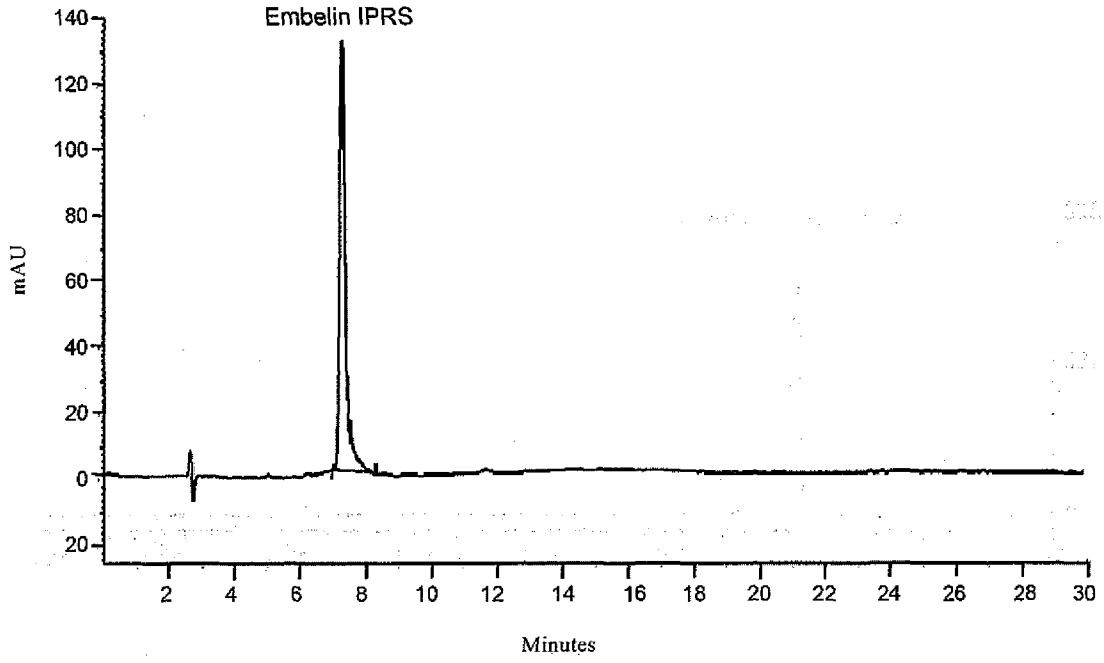


HPLC Chromatogram of vasicine hydrochloride IPRS

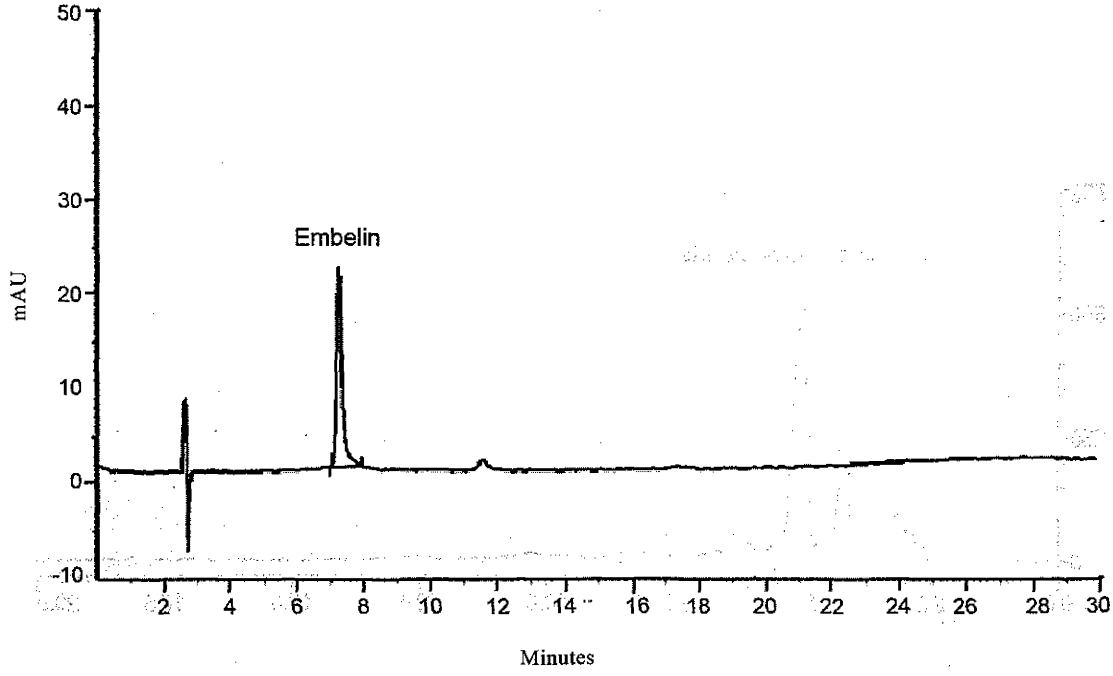


HPLC Chromatogram of Vasaka Extract

Vidanga

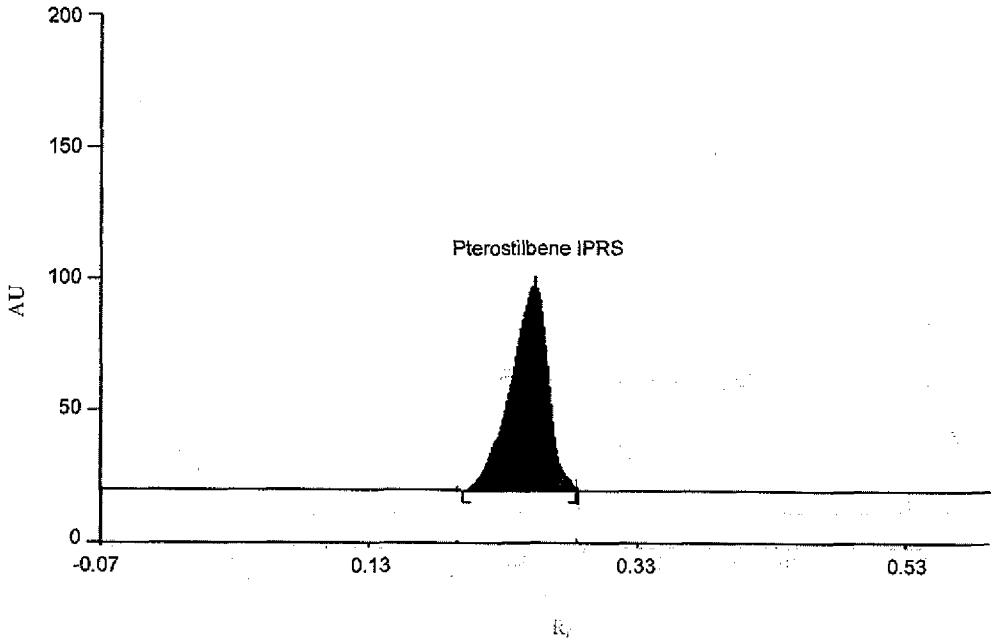


HPTLC Chromatogram of embelin IPRS

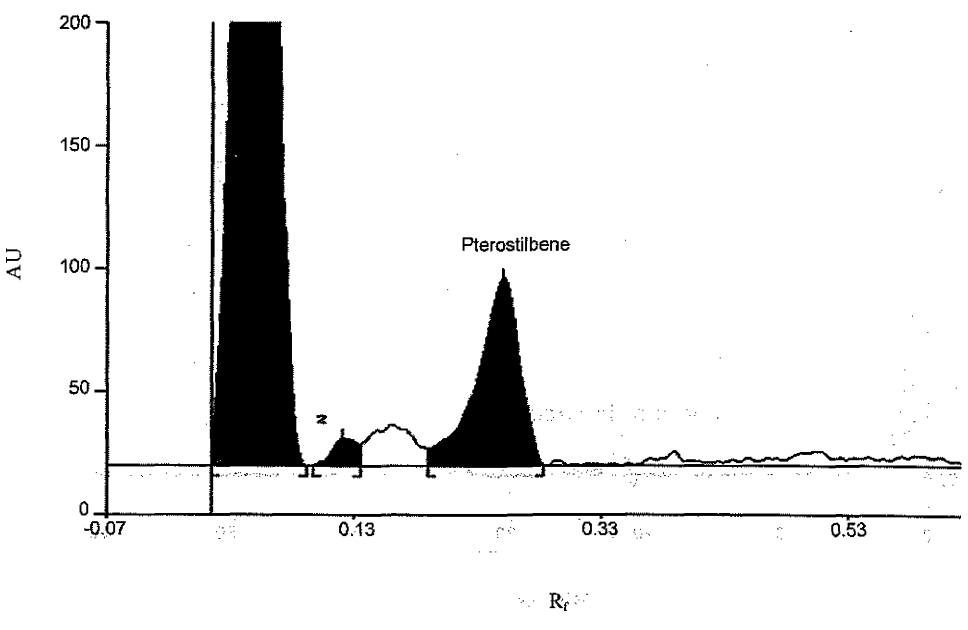


HPLC Chromatogram of Vidanga

Vijayasara

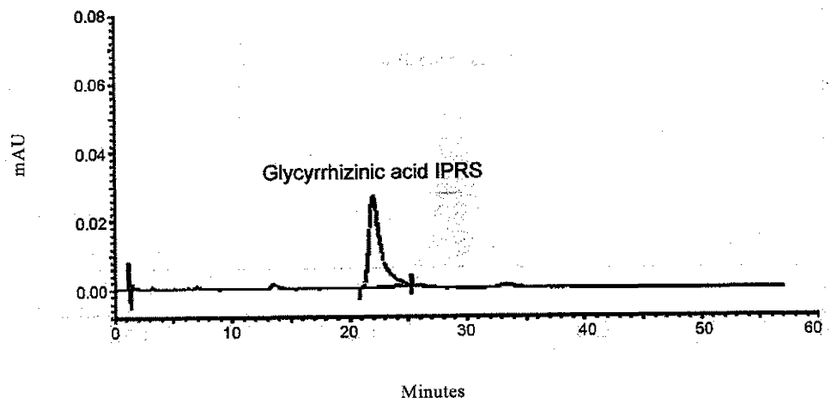


HPTLC Chromatogram of pterostilbene IPRS

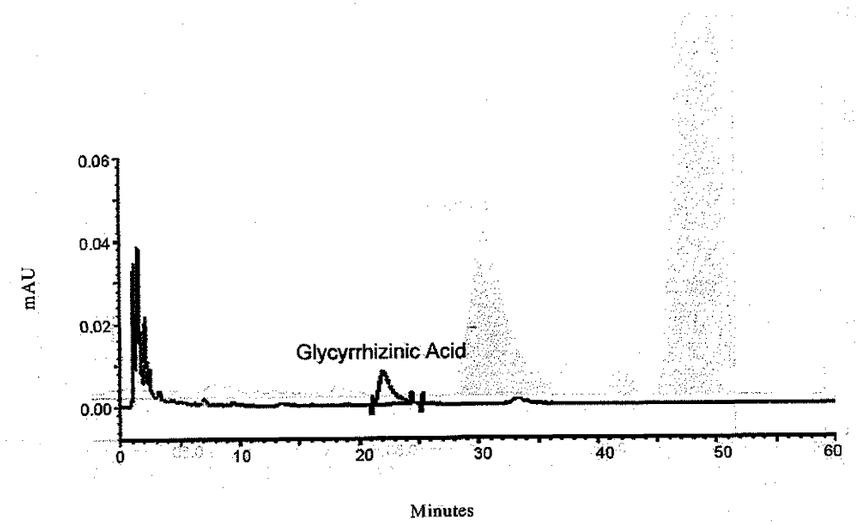


HPTLC Chromatogram of Vijayasara

Yasti

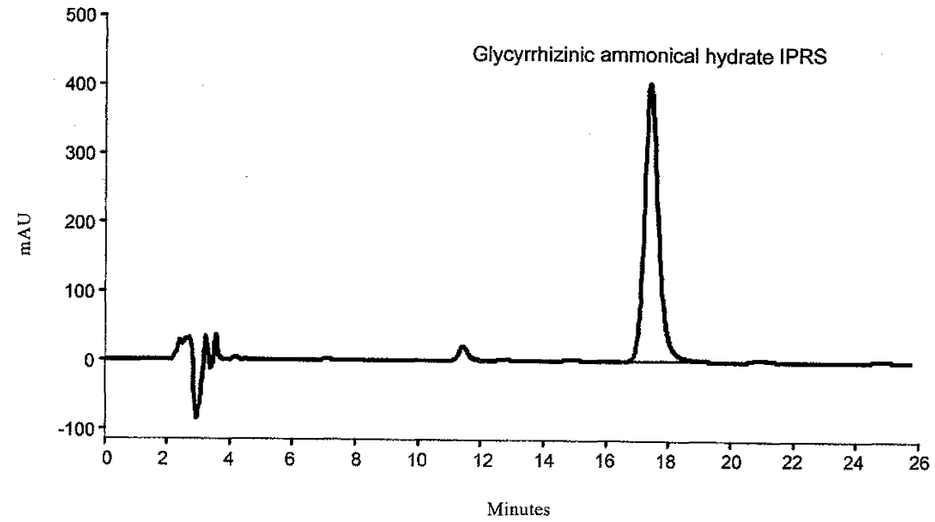


HPLC Chromatogram of glycyrrhizinic acid IPRS

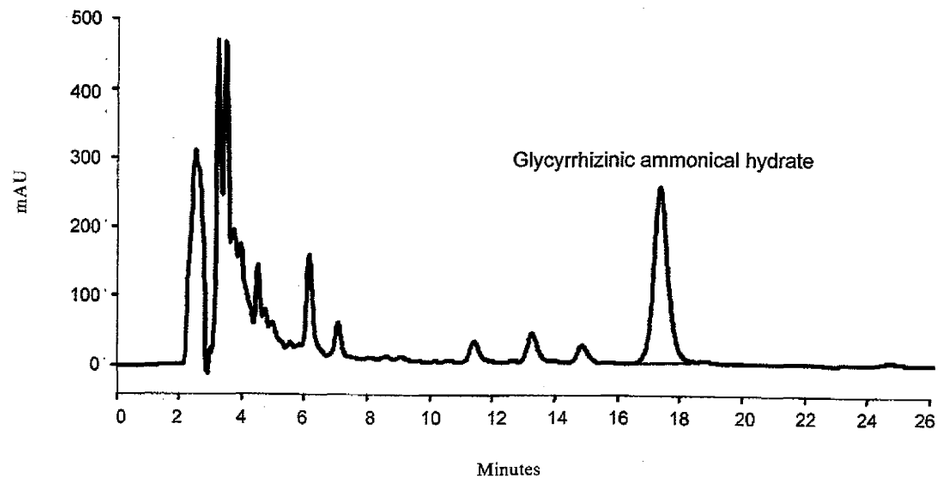


HPLC Chromatogram of Yasti

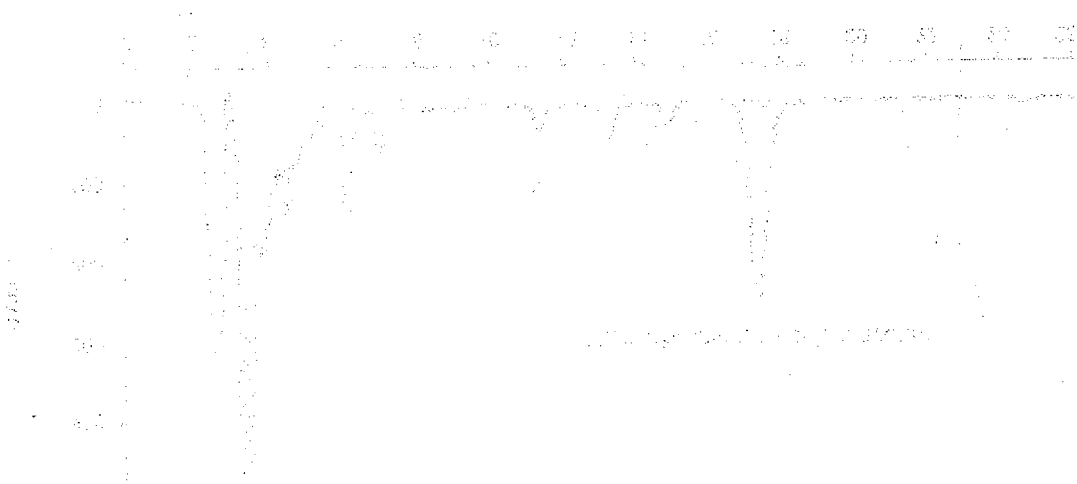
Yasti Dry Extract

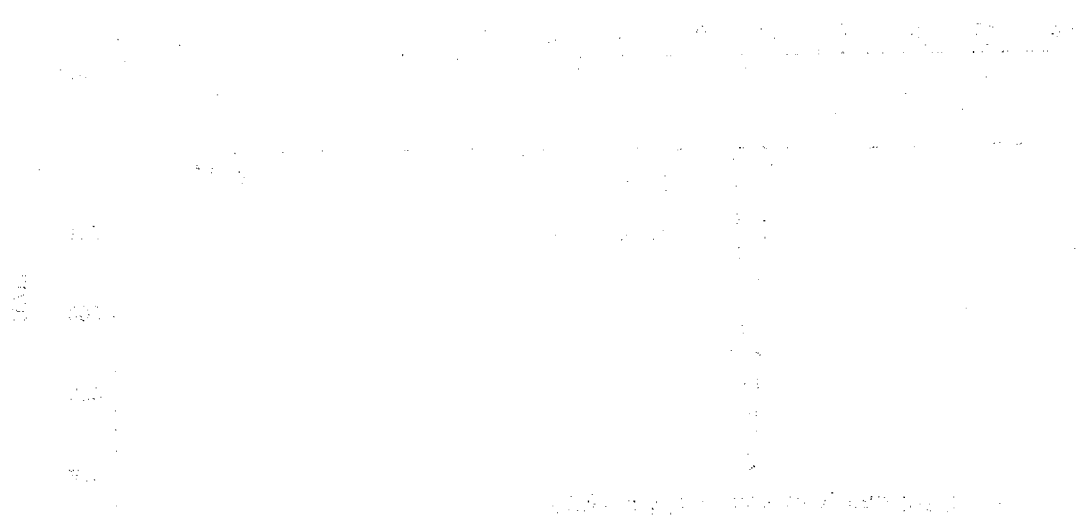


HPLC Chromatogram of glycyrrhizinic ammonical hydrate IPRS



HPLC Chromatogram of Yasti Dry Extract





4. REAGENTS AND SOLUTIONS

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4.1. Buffer Solutions

A. Standard Buffer Solutions

Standard Buffer Solutions are solutions of standard pH. They are used for reference purposes in pH measurements and for carrying out many pharmacopoeial tests which require adjustments to or maintenance of a specified pH. They may be prepared by the methods described below. The preparation of special buffer solutions is described in the sections in which their use is specified as in the *microbiological assay of antibiotics* or in the individual monographs where the use of such solutions is indicated.

The reagents required for the preparation of standard buffer solutions are described in Appendix 4.2. All the crystalline reagents except boric acid should be dried at 110° to 120° for 1 hour before use. *Carbon dioxide-free water* should be used for preparing buffer solutions and wherever *water* is mentioned for preparation of such solutions the use of *carbon dioxide-free water* is implied. The prepared solutions should be stored in chemically resistant, glass-stoppered bottles of alkakli-free glass and used within 3 months of preparation. Any solution which has become cloudy or shows any other evidence of deterioration should be discarded.

Standard buffer solutions for various ranges of pH values 1.2 to 10.0 may be prepared by appropriate combinations of *0.2 M hydrochloric acid* or *0.2 M sodium hydroxide* and of solutions described below, used in the proportions shown in the accompanying tables. The standard pH values given in the tables and elsewhere in the Appendix are considered to be reproducible within ± 0.02 Unit at 25°.

1. Boric Acid and Potassium Chloride, 0.2 M: Dissolve 12.366 g of *boric acid* and 14.911 g of *potassium chloride* in *water* and dilute with *water* to 1000 ml.

2. Disodium Hydrogen Phosphate, 0.2 M: Dissolve 71.630 g of *disodium hydrogen phosphate* in *water* and dilute with *water* to 1000 ml.

3. Hydrochloric Acid, 0.2 M: *Hydrochloric acid* diluted with *water* to contain 7.292 g of HCl in 1000 ml. Standardise as directed in Appendix 4.4.

4. Potassium Chloride, 0.2 M: Dissolve 14.911 g of *potassium chloride* in *water* and dilute with *water* to 1000 ml.

5. Potassium Dihydrogen Phosphate, 0.2 M: Dissolve 27.218 g of *potassium dihydrogen phosphate* in *water* and dilute with *water* to 1000 ml.

6. Potassium Hydrogen Phthalate, 0.2 M: Dissolve 40.846 g of *potassium hydrogen phthalate* in *water* and dilute with *water* to 1000 ml.

7. Sodium Hydroxide, 0.2 M: Dissolve *sodium hydroxide* in *water* to produce a 40 to 60 per cent w/v solution and allow to stand. Taking precautions to avoid absorption of carbon dioxide, siphon off the clear supernatant liquid and dilute with *carbon dioxide-free water*, a suitable volume of the liquid to contain 8.0 g of NaOH in 1000 ml. Standardise as directed in Appendix 4.4.

NOTE — 0.2 M Sodium hydroxide must not be used later than one month after preparation.

Composition of Standard Buffer Solutions

Hydrochloric Acid Buffer: Place 50.0 ml of the *0.2 M potassium chloride* in a 200 ml volumetric flask, add the specified volume of *0.2 M hydrochloric acid* (see Table 1) and then add *water* to volume.

Table 1

pH	0.2 M HCl, ml	pH	0.2 M HCl, ml
1.2	85.0	1.8	20.4
1.3	67.2	1.9	16.2
1.4	53.2	2.0	13.0
1.5	41.4	2.1	10.2
1.6	32.4	2.2	7.8
1.7	26.0		

Acid Phthalate Buffer: Place 50.0 ml of *0.2 M potassium hydrogen phthalate* in a 200 ml volumetric flask, add the specified volume of *0.2 M hydrochloric acid* (see Table 2) and then add *water* to volume.

Table 2

pH	0.2 M HCl, ml	pH	0.2 M HCl, ml
2.2	49.5	3.2	15.7
2.4	42.2	3.4	10.4
2.6	35.4	3.6	6.3
2.8	28.9	3.8	2.9
3.0	22.3	4.0	0.1

Neutralised Phthalate Buffer; Phthalate Buffer: Place 50.0 ml of *0.2 M potassium hydrogen phthalate* in a 200 ml volumetric flask, add the specified volume of *0.2 M sodium hydroxide* (see Table 3) and then add *water* to volume.

Table 3

pH	0.2 M NaOH, ml	pH	0.2 M NaOH, ml
4.2	3.0	5.2	28.8
4.4	6.6	5.4	34.1
4.6	11.1	5.6	38.8
4.8	16.5	5.8	42.3
5.0	22.6		

Phosphate Buffer: Place 50.0 ml of *0.2 M potassium dihydrogen phosphate* in a 200 ml volumetric flask, add the

specified volume of *0.2 M sodium hydroxide* (see Table 4) and then add *water* to volume.

Table 4

pH	0.2 M NaOH, ml	pH	0.2 M NaOH, ml
5.8	3.6	7.0	29.1
6.0	5.6	7.2	34.7
6.2	8.1	7.4	39.1
6.4	11.6	7.6	42.4
6.6	16.4	7.8	44.5
6.8	22.4	8.0	46.1

Alkaline Borate Buffer: Place 50.0 ml of *0.2 M boric acid* and *potassium chloride* in a 200 ml volumetric flask, add the specified volume of *0.2 M sodium hydroxide* (see Table 5) and then add *water* to volume.

Table 5

pH	0.2 M NaOH, ml	pH	0.2 M NaOH, ml
8.0	3.9	9.2	26.4
8.2	6.0	9.4	32.1
8.4	8.6	9.6	36.9
8.6	11.8	9.8	40.6
8.8	15.8	10.0	43.7
9.0	20.8		

NOTE — In this Pharmacopoeia standard buffer solutions are referred to as "buffer solution pH...." *or* "buffer pH...."

B. Other Buffer solutions

Acetate Buffer pH 2.8: Dissolve 4 g of *anhydrous sodium acetate* in about 840 ml of *water*, add sufficient *glacial acetic acid*, adjusted to pH 2.8 (about 155 ml) and dilute with *water* to 1000 ml.

Acetate Buffer pH 3.4: Mix 50 ml of *0.1 M sodium acetate* with 950 ml of *0.1 M acetic acid*.

Acetate Buffer pH 3.5: Dissolve 25 g of *ammonium acetate* in 25 ml of *water* and add 38 ml of *7 M hydrochloric acid*. Adjusted to pH 3.5 with either *2 M hydrochloric acid* or *6 M ammonia* and dilute with *water* to 100 ml.

Acetate Buffer pH 3.7: Dissolve 10 g of *anhydrous sodium acetate* in 300 ml of *water*, adjusted to pH 3.7 with *glacial acetic acid* and dilute with *water* to 1000 ml. Before use adjusted to pH 3.7, if necessary, with *glacial acetic acid* or *anhydrous sodium acetate*, as required.

Acetate Buffer pH 4.0: Place 2.86 ml of *glacial acetic acid* and 1.0 ml of a 50 per cent w/v solution of *sodium hydroxide* in a 1000 ml volumetric flask, add *water* to volume and mix. Adjusted to pH, if necessary.

Acetate Buffer pH 4.4: Dissolve 136 g of *sodium acetate* and 77 g of *ammonium acetate* in *water* and dilute with *water* to 1000 ml. Add 250 ml of *glacial acetic acid* and mix.

Acetate Buffer pH 4.6: Dissolve 5.4 g of *sodium acetate* in 50 ml of *water*, add 2.4 ml of *glacial acetic acid* and dilute with *water* to 100 ml. Adjust the pH, if necessary.

Acetate Buffer pH 4.7: Dissolve 8.4 g of *sodium acetate* and 3.35 ml of *glacial acetic acid* in sufficient *water* to produce 1000 ml. Adjusted to pH, if necessary.

Acetate Buffer pH 5.0: Dissolve 13.6 g of *sodium acetate* and 6 ml of *glacial acetic acid* in sufficient *water* to produce 1000 ml. Adjust the pH, if necessary.

Acetate Buffer pH 5.5: Dissolve 272 g of *sodium acetate* in 500 ml of *water* by heating to 35°, cool and add slowly 50 ml of *glacial acetic acid* and sufficient *water* to produce 1000 ml. Adjusted to pH, if necessary.

Acetate Buffer pH 6.0: Dissolve 100 g of *ammonium acetate* in 300 ml of *water*, add 4.1 ml of *glacial acetic acid*, adjusted to pH, if necessary, using *10 M ammonia* or *5 M acetic acid* and dilute with *water* to 500 ml.

Acetate Buffer Solution: Dissolve 14 g of *potassium acetate* and 20.5 ml of *glacial acetic acid* in sufficient *water* to produce 1000 ml.

Acetate-Edetate Buffer pH 5.5: Dissolve 250 g of *ammonium acetate* and 15 g of *disodium edetate* in 400 ml of *water* and add 125 ml of *glacial acetic acid* in sufficient water to produce 1000 ml.

Acetic Acid-Ammonium Acetate Buffer: Dissolve 77.1 g of *ammonium acetate* in *water*, add 57 ml of *glacial acetic acid* and dilute with *water* to 1000 ml.

Acetic Ammonia Buffer pH 3.7, Ethanolic: To 15 ml of *5 M acetic acid* add 60 ml of *ethanol (95 per cent)* and 24 ml of *water*. Adjusted to pH 3.7 with *10 M ammonia* and dilute with *water* to 100 ml.

Acetone Solution, Buffered: Dissolve 8.15 g of *sodium acetate* and 42 g of *sodium chloride* in *water*, add 68 ml of *0.1 M hydrochloric acid* and 150 ml of *acetone* and dilute with *water* to 500 ml.

Albumin Phosphate Buffer pH 7.2; Phosphate-albumin Buffered Saline pH 7.2: Dissolve 10.75 g of *disodium hydrogen phosphate*, 7.6 g of *sodium chloride* and 10 g of *bovine albumin* in sufficient *water* to produce 1000 ml. Before use, adjusted to pH 7.2 with 2 M *sodium hydroxide* or a 10 per cent w/v solution of *phosphoric acid* as required.

Ammonia-Ammonium Chloride Buffer: Dissolve 67.5 g of *ammonium chloride* in about 200 ml of *water*, add 570 ml of *strong ammonia solution* and dilute with *water* to 1000 ml.

Ammonia Buffer pH 9.5: Dissolve 33.5 g of *ammonium chloride* in 150 ml of *water*, and 42 ml of *10 M ammonia* and dilute with *water* to 250 ml.

Store in polyethylene containers.

Ammonia Buffer pH 10.0; Ammonium Chloride Buffer Solution pH 10.0: Dissolve 5.4 g of *ammonium chloride* in 20 ml of *water*, add 35 ml of *10 M ammonia* and dilute with *water* to 100 ml.

Ammonia Buffer pH 10.9: Dissolve 67.5 g of *ammonium chloride* in sufficient *10 M ammonia* to produce 1000 ml.

Barbitone Buffer pH 7.4: Mix 50 ml of solution containing 1.944 per cent w/v of *sodium acetate* and 2.946 per cent w/v of *barbitone sodium* with 50.5 ml of *0.1 M hydrochloric acid*, add 20 ml of an 8.5 per cent w/v solution of *sodium chloride* and dilute with *water* to 250 ml.

Barbitone Buffer pH 8.4: Dissolve 8.25 g of *barbitone sodium* in sufficient *water* to produce 1000 ml.

Barbitone Buffer pH 8.6, Mixed; Barbitone Buffer pH 8.6: Dissolve 1.38 g of *barbitone*, 8.76 g of *barbitone sodium* and 0.38 g of *calcium lactate* in sufficient *water* to produce 1000 ml.

Boric Buffer pH 8.0, 0.0015 M; Borate Buffer pH 8.0, 0.0015 M: Dissolve 0.572 g of *disodium tetraborate* and 2.94 g of *calcium chloride* in 800 ml of *water*. Adjusted to pH 8.0 with *1 M hydrochloric acid* and dilute to 1000.0 ml with *water*.

Boric Buffer pH 9.0; Borate Buffer pH 9.0: Dissolve 6.20 g of *boric acid* in 500 ml of *water*, adjusted to pH 9.0 with *1 M sodium hydroxide* (about 41.5 ml) and dilute with *water* to 1000.0 ml.

Boric Buffer pH 10.4; Borate Buffer pH 10.4: Dissolve 24.64 g of *boric acid* in 900 ml of *water*. Adjusted to pH 10.4 with *10 M sodium hydroxide* and dilute with *water* to 1000 ml.

Buffer Solution pH 2.5: To 25.0 ml of *0.2 M potassium hydrogen phthalate* add 37.0 ml of *0.1 M hydrochloric acid* and dilute with sufficient *water* to produce 100.0 ml.

Buffer Solution pH 7.2: To 250.0 ml of *0.2 M potassium dihydrogen phosphate* add 175.0 ml of *0.2 M sodium hydroxide*. Dilute to 1000.0 ml with *water*.

Carbonate Buffer pH 9.7: Dissolve 8.4 g of *sodium bicarbonate* and 10.6 g of *sodium carbonate* in sufficient *water* to produce 500 ml.

Chloride Buffer pH 2.0: Dissolve 6.57 g of *potassium chloride* in *water*, add 119.0 ml of *0.1 M hydrochloric acid* and dilute with *water* to 1000 ml.

Citrate Buffer: Dissolve 0.5 g of *citric acid monohydrate* and 0.4 g of *dibasic sodium phosphate* in sufficient *water* to produce 1000 ml.

Citrate Buffer pH 3.0, 0.25 M: Dissolve 4.8 g of *citric acid* in 80 ml of *water*. Adjusted the pH to 3.0 with *1 M sodium hydroxide* and dilute to 100 ml with *water*.

Citro-phosphate Buffer pH 5.0: Mix 48.5 ml of *0.1 M citric acid* with sufficient *0.2 M disodium hydrogen phosphate* to produce 100 ml.

Citro-phosphate Buffer pH 6.0: Mix 36.8 ml of a 2.1 per cent w/v solution of *citric acid* with 63.2 ml of a 7.15 per cent w/v solution of *disodium hydrogen phosphate*.

Citro-phosphate Buffer pH 7.0: Mix 17.6 ml of a 2.1 per cent w/v solution of *citric acid* with 82.4 ml of a 7.15 per cent w/v solution of *disodium hydrogen phosphate*.

Citro-phosphate Buffer pH 7.2: Mix 13.0 ml of a 2.1 per cent w/v solution of *citric acid* with 87.0 ml of a 7.15 per cent w/v solution of *disodium hydrogen phosphate*.

Citro-phosphate Buffer pH 7.6: Dissolve 1.33 g of *citric acid* and 67.1 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Cupric Sulphate Solution pH 2.0, Buffered: Mix 5.3 ml of *0.2 M hydrochloric acid* and 25 ml of *0.2 M potassium chloride*, add 4 ml of a 0.393 per cent w/v solution of *cupric sulphate* and dilute to 100 ml of *water*.

Cupric Sulphate Solution pH 4.0, Buffered: Dissolve 0.25 g *cupric sulphate* and 4.5 g of *ammonium acetate* in sufficient *water* to produce 100 ml.

Cupric Sulphate Solution pH 5.2, Buffered: Dissolve 1.522 g of *anhydrous disodium hydrogen phosphate* in sufficient *water* to produce 53.6 ml and add a 2.1 per cent solution of *citric acid* until the pH of the solution is between 5.15 and 5.25 (about 46 ml). Mix 98.5 ml of the resulting solution with 1.5 ml of a 0.393 per cent solution of *cupric sulphate*.

Diethanolamine Buffer pH 10.0: Dissolve 96.4 g of *diethanolamine* in sufficient *water* to produce 400 ml. Add 0.5 ml of an 18.6 per cent w/v solution of *magnesium chloride*, adjusted to pH 10.0 with *1 M hydrochloric acid* and dilute with *water* to 500.0 ml.

Diethylammonium Phosphate Buffer Solution pH 6.0: Dilute 68 ml of *orthophosphoric acid* to 500 ml with *water*. To 25 ml of this solution add 450 ml of *water* and 6 ml of *diethylamine*, adjusted to pH 6 ± 0.05, if necessary, using *diethylamine* or *orthophosphoric acid* and dilute to 500.0 ml with *water*.

Glycine Buffer pH 11.3: Mix a solution containing 0.75 per cent w/v of *glycine* and 0.58 per cent w/v of *sodium chloride* with an equal volume of *0.1 M sodium hydroxide*. Adjusted the pH if necessary.

Glycine Buffer Solution: Mix 42 g of *sodium bicarbonate* and 50 g of *potassium bicarbonate* with 180 ml of *water* and

add a solution containing 37.5 g of *glycine* and 15 ml of *strong ammonia* in 180 ml of *water*. Dilute with *water* to 500.0 ml and stir until solution is complete.

HEPES Buffer Solution pH 7.5: Dissolve 2.38 g of *2[4-(hydroxyethyl)piperazin-1-yl]ethanesulphonic acid* in about 90 ml of *water*. Adjusted the pH to 7.5 with *sodium hydroxide solution*. Dilute to 100 ml with *water*.

Imidazole Buffer pH 6.5: Dissolve 6.81 g of *imidazole* and 1.23 g of *magnesium sulphate* in 752 ml of *0.1 M hydrochloric acid*, adjust the pH, if necessary and dilute with *water* to produce 1000 ml.

Imidazole Buffer pH 7.3: Dissolve 3.4 g of *imidazole* and 5.8 g of *sodium chloride* in *water*, add 18.6 ml of *1 M hydrochloric acid* and dilute with *water* to produce 1000 ml. Adjusted to pH, if necessary.

Imidazole buffer solution pH 7.3 for Thrombin Assay: Dissolve 0.34 g of *imidazole salt*, 0.58 g of *sodium chloride*, 0.37 g of *calcium chloride*, 1.86 ml of *1M hydrochloric acid* in 40 ml of distilled *water* and make upto 100 ml and adjusted to pH 7.3.

Palladium Chloride Solution, Buffered: To 0.5 g of *palladium chloride* add 5 ml of *hydrochloric acid* and warm on a water-bath. Add 200 ml of hot *water* in small portions with continued heating until solution is complete. Cool and dilute with sufficient *water* to produce 250.0 ml. To 50.0 ml of the resulting solution add 10.0 ml of *1 M sodium acetate*, 9.6 ml of *1 M hydrochloric acid* and sufficient *water* to produce 100.0 ml.

Phosphate Buffer pH 2.0: Dissolve 0.136 g of *potassium dihydrogen phosphate* in 800 ml of *water*, adjust the pH to 2.0 with *hydrochloric acid* and add sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 2.5: Dissolve 100 g of *potassium dihydrogen phosphate* in 800 ml of *water*, adjust the pH to 2.5 with *hydrochloric acid* and add sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 3.0: Dissolve 1.36 g of *potassium dihydrogen orthophosphate* and 2 ml of *triethylamine* in 800 ml of *water*, adjust the pH to 3.0 with *orthophosphoric acid* and add sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 3.0, 0.1 M. Dissolve 12.0 g of *anhydrous sodium dihydrogen phosphate* in *water*, adjusted to pH with *dilute phosphoric acid* and dilute to 1000.0 ml with *water*.

Phosphate Buffer pH 3.2. To 900 ml of a solution prepared by dissolving 4 g of *sodium dihydrogen phosphate* in 1000 ml of *water* and 100 ml of a solution prepared by diluting 2.5 g of *phosphoric acid* in 1000.0 ml of *water*. Adjusted to pH 3.2 if necessary with *1M sulphuric acid* or *1M sodium hydroxide* as required.

Phosphate Buffer pH 3.5, 0.02 M: Dissolve 2.7 g of *potassium dihydrogen phosphate* in 900 ml of *water*. Adjust the pH to 3.5 with *phosphoric acid*. Dilute to 1000.0 ml with *water*.

Phosphate Buffer pH 3.6: Dissolve 0.900 g of *anhydrous disodium hydrogen phosphate* and 1.298 g of *citric acid monohydrate* in sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 4.0, Mixed: Dissolve 5.04 g *disodium hydrogen phosphate* and 3.01 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml. Adjust the pH with *glacial acetic acid*.

Phosphate buffer pH 4.4. Dissolve 7.8 g of *monobasic sodium phosphate* in 900 ml of *water*, adjusted to pH 4.4 with *10 M sodium hydroxide* or *orthophosphoric acid* and diluting with *water* to 1000.0 ml.

Phosphate Buffer pH 4.75: Dilute 100 ml of *0.5M potassium dihydrogen orthophosphate* to 800 ml with *water*, adjusted to pH 4.75 with *0.1M sodium hydroxide* and dilute to 1000 ml with *water*.

Phosphate Buffer pH 4.9: Dissolve 40 g of *sodium dihydrogen phosphate* and 1.2 g of *sodium hydroxide* in sufficient *water* to produce 100 ml. If necessary, adjusted to pH with *1 M sulphuric acid* or *1 M sodium hydroxide* as required.

Phosphate Buffer pH 5.0: Dissolve 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and adjusted to pH 5.0 with *10 M potassium hydroxide*.

Phosphate Buffer pH 5.5, Mixed

SOLUTION I — Dissolve 13.61 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml.

SOLUTION II — Dissolve 35.81 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Mix 96.4 ml of solution I with 3.6 ml of solution II.

Phosphate Buffer pH 6.3, 0.1 M: Prepare 0.1 M monobasic sodium phosphate and adjust with 10 M sodium hydroxide to a pH 6.3 ± 0.2.

Phosphate Buffer pH 6.5: Dissolve 60.5 g of *disodium hydrogen phosphate* and 46 g of *potassium dihydrogen phosphate* in *water*, add 100 ml of *0.02 M disodium edetate* and 20 mg of *mercuric chloride* and dilute with *water* to produce 1000 ml.

Phosphate Buffer pH 6.5, 0.1M: Dissolve 13.80 g of *sodium dihydrogen phosphate monohydrate* in 900 ml of *water*. Adjusted to pH 6.5 with *10 M sodium hydroxide* and dilute with *water* to 1000 ml.

Phosphate buffer pH 6.5, 0.05 M. Dissolve 6.90 g of *sodium dihydrogen phosphate monohydrate* in 900 ml of *water*. Adjusted to pH 6.5 with *10 M sodium hydroxide* and dilute with *water* to 1000 ml.

Phosphate Buffer pH 6.7, 0.1 M: Dissolve 15.6 g of *sodium dihydrogen phosphate* in *water* and dilute to 1000 ml with the same solvent. Dissolve 17.8 g of *disodium hydrogen phosphate dihydrate* in *water* and dilute to 1000 ml with the same solvent. Mix the solutions, check the pH and adjusted to pH 6.7, if necessary.

Phosphate Buffer pH 6.8, Mixed: Dissolve 28.80 g of *disodium hydrogen phosphate* and 11.45 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 6.8, 0.2 M Mixed: Dissolve 13.872 g of *potassium dihydrogen phosphate* and 35.084 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Store in a cold place.

Phosphate Buffer pH 7.0, Mixed: Dissolve 0.50 g of *anhydrous disodium hydrogen phosphate* 0.301 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 7.0 with Azide, Mixed: To 1000 ml of a solution containing 1.8 per cent w/v of *disodium hydrogen phosphate* and 2.3 per cent w/v of *sodium chloride*, add sufficient of a solution containing 0.78 per cent w/v of *sodium dihydrogen phosphate* and 2.3 per cent w/v of *sodium chloride* (about 280 ml) to produce a pH of 7.0. Dissolve sufficient *sodium azide* in the resulting solution to give a 0.02 per cent w/v solution.

Phosphate Buffer pH 7.0, 0.067 M Mixed: Dissolve 3.532 g of *potassium dihydrogen phosphate* and 14.542 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 7.0, 0.1 M Mixed; Phosphate Buffer pH 7.0, 0.1 M: Dissolve 1.361 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 100 ml and adjusted to pH using 3.5 per cent w/v solution of *disodium hydrogen orthophosphate*.

Phosphate Buffer pH 7.5: Dissolve 6.8 g of *potassium dihydrogen orthophosphate* and 1.56 g of *sodium hydroxide* in 900 ml of *water*, adjusted to pH 7.5 with *sodium hydroxide solution* and dilute with *water* to produce 1000 ml.

Phosphate Buffer pH 7.5, 0.05 M: Dissolve 0.89 g of *disodium hydrogen phosphate dihydrate* in about 80 ml of water. Adjusted to pH 7.5 with an 8.5 per cent *v/v* solution of *phosphoric* acid and dilute to 100.0 ml with *water*.

Phosphate Buffer pH 7.5, 0.2 M: Dissolve 27.2 g of *potassium dihydrogen phosphate* with 930 ml of *water* adjust the pH 7.5 with 0.3 per cent w/v solution of *potassium hydroxide* and add sufficient *water* to produce 1000 ml.

Phosphate Buffer pH 7.5, 0.33 M Mixed

SOLUTION I — Dissolve 119.31 g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

SOLUTION II — Dissolve 45.36 g of *potassium dihydrogen phosphate* in sufficient *water* to produce 1000 ml.

Mix 85 ml of solution I and 15 ml of solution II and adjust the pH if necessary.

Phosphate Buffer pH 8.0, 0.02 M: Mix 50 ml of *0.2 M potassium dihydrogen phosphate* with 46.8 ml of *0.2 M sodium hydroxide* and add sufficient *water* to produce 500 ml.

Phosphate Buffer, 0.025 M Standard: Dissolve 3.40 g of *potassium dihydrogen phosphate* and 3.55 g of *anhydrous disodium hydrogen phosphate*, both previously dried at 110° to 130° for 2 hours, in sufficient *water* to produce 1000 ml.

Phosphate Buffer, 0.05 M: Dissolve 6.8 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 1000 ml.

Saline, Phosphate-buffered: Dissolve 2.5 g of *sodium dihydrogen phosphate*, 2.523 g of *disodium hydrogen phosphate* and 8.2 g of *sodium chloride* in sufficient *water* to produce 1000 ml.

Saline pH 6.4, Phosphate-buffered: Dissolve 1.79 g of *disodium hydrogen phosphate*, 1.36 g of *potassium dihydrogen phosphate* and 7.02 g of *sodium chloride* in sufficient *water* to produce 1000 ml.

Saline pH 7.4, Phosphate-buffered: Dissolve 2.38 g of *disodium hydrogen phosphate*, 0.19 g of *potassium dihydrogen phosphate* and 8.0 g of *sodium chloride* in sufficient *water* to produce 1000 ml. Adjusted to pH, if necessary.

Sodium Acetate Buffer Solution pH 4.5: Dissolve 63 g of *anhydrous sodium acetate* in *water*, add 90 ml *acetic acid* and adjusted to pH 4.5, and dilute to 1000 ml with *water*.

Sulphate Buffer pH 2.0: Dissolve 132.1 gm of *ammonium sulphate* in *water* and dilute to 500 ml with the *water* (solution I). Dissolve 14 ml of *sulphuric acid* into about 400 ml of *water* with constant cooling and stirring. Allow to cool and dilute to 500 ml with *water* (solution II). Mix equal volumes of solutions I and II. Adjusted to pH, if necessary.

Tris-Acetate Buffer pH 8.5: Dissolve 0.294 g of *calcium chloride* and 12.11 g of *tris(hydroxymethyl)aminomethane* in *water*. Adjusted to pH with *5 M acetic acid* and dilute to 1000.0 ml with *water*.

Tris-Chloride Buffer pH 7.4: Dissolve 7.27 g of *tris(hydroxymethyl)methylamine* and 5.27 g of *sodium chloride*, adjusted to pH, if necessary and dilute with *water* to produce 1000 ml.

Tris-EDTA BSA Buffer Solution pH 8.4: Dissolve 6.1 g of *tris (hydroxyl)aminomethane*, 2.8 g of *sodium edetate*, 10.2 g of *sodium chloride* and 10 g of *bovine albumin* in *water*, adjusted to pH 8.4 using *hydrochloric acid* and dilute with *water* to produce 1000 ml.

Tris(hydroxymethyl)aminomethane Buffer pH 7.4: Dissolve 30.3 g of *tris(hydroxymethyl)aminimethane* in approximately 200 ml of *water*. Add 183 ml of 1 *M hydrochloric acid*. Dilute to 500.0 ml with *water*.

NOTE — The pH is 7.7 to 7.8 at room temperature and 7.4 at 37°. This solution is stable for several months at 4°.

Tris-hydrochloride Buffer pH 8.0, 1 M: Dissolve 121.1 g of *tris(hydroxymethyl)aminomethane* and 1.47 g of *calcium chloride* in 900 ml of *water*. Adjusted to pH 8.0 with *hydrochloric acid* and dilute to 1000 ml with *water*.

Tris(hydroxymethyl)aminomethane Buffer pH 8.1: Dissolve 2.9 g of *calcium chloride* with 400 ml of *tris(hydroxymethyl) aminomethane solution* adjusted to pH with *1 M hydrochloric acid* and dilute with *water* to produce 1000 ml.

4.2 General Reagents

Acetal; Acetaldehyde diethyl acetal, 1,1-Diethoxyethane; $C_6H_{14}O_2 = 118.2$

Analytical reagent grade of commerce.

Clear, colourless, volatile liquid; miscible with water and with ethanol (95 per cent); bp, about 103°; wt. per ml, about 0.824 g.

Acetaldehyde: $CH_3CHO = 44.05$

General laboratory reagent grade of commerce.

Clear, colourless, volatile liquid with an acrid, penetrating odour; bp, about 21°; wt. per ml, about 0.79 g.

Complies with the following test.

ACIDITY — To 10.0 g, add sufficient *carbon dioxide-free water* to produce 50 ml and titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator; not more than 5.0 ml of *0.1 M sodium hydroxide* is required.

Acetylacetamide; 3-Oxobutanamide: $C_4H_7NO_2 = 101.1$

General laboratory reagent grade of commerce.

mp, about 53° to 56°.

***N*-Acetylneuraminic acid**: $C_{11}H_{19}NO_9 = 309.3$

White acicular crystals, soluble in *water* and in *methanol*, slightly soluble in *ethanol*, practically insoluble in *acetone*.

$[\alpha]_D^{20}$: about -36, determined on a 1 per cent w/v solution.; mp, about 186°, with decomposition.

Acetic Acid: Analytical reagent grade of commerce; contains approximately 33 per cent w/w of CH_3COOH. Dilute 315 ml of *glacial acetic acid* to 1000 ml with *water*.

Acetic Acid, x M: Solutions of any molarity x M may be prepared by diluting 57 x ml (60 x g) of *glacial acetic acid* to 1000 ml with *water*.

Acetic Acid, Dilute: Contains approximately 6 per cent w/w of CH_3COOH. Dilute 57 ml of *glacial acetic acid* to 1000 ml with *water*.

Acetic Acid, Glacial: $CH_3COOH = 60.05$

Analytical reagent grade of commerce.

Clear, colourless liquid; odour, pungent; about 17.5 M in strength; congealing temperature, not lower than 14.8°; bp, about 118°; wt. per ml, about 1.047 g.

Acetic Acid, Glacial, Anhydrous; Anhydrous Acetic Acid: $CH_3COOH = 60.05$

Glacial acetic acid of commerce for use in non-aqueous titrations.

Complies with the following test.

WATER (2.3.43) — Not more than 0.4 per cent w/w. If the water content is greater than 0.4 per cent, it may be adjusted by adding the calculated amount of *acetic anhydride*.

Acetic Acid Sp.: *Acetic acid* which complies with the following test.

Make 25 ml alkaline with dilute *ammonia solution Sp.*, add 1 ml of *potassium cyanide solution Sp.*, dilute to 50 ml with *water* and add 2 drops of *sodium sulphide solution*; no darkening is produced.

Acetic Acid Sp., Dilute: *Dilute acetic acid* which complies with the following test.

Evaporate 20 ml in a porcelain dish nearly to dryness on a water-bath. Add to the residue 2 ml of the sample and dilute with *water* to 25 ml, add 10 ml of *hydrogen sulphide solution*; any dark colour produced is not more intense than that of a solution consisting of 2 ml of the sample and 4.0 ml of the *standard lead solution (2 ppm Pb)* diluted to 25 ml with *water*.

Acetic Anhydride: $(CH_3CO)_2O = 102.09$

Analytical reagent grade of commerce.

Clear, colourless liquid; odour, pungent; wt. per ml, about 1.08 g; distillation range, 136° to 142°.

Acetic Anhydride Solution. Dilute 25 ml of *acetic anhydride* in *anhydrous pyridine* and dilute to 100.0 ml with *anhydrous pyridine*.

Store protected from light and air.

Acetic Anhydride-Dioxan Solution: Add 1 ml of *acetic anhydride* to 50 ml of *dioxan*.

Acetone; 2-Propanone: $(CH_3)_2CO = 58.08$

Analytical reagent grade of commerce.

Clear, colourless, volatile liquid; odour, characteristic; flammable; bp, about 56°; wt. per ml, about 0.79 g.

Acetone, Dry: *Acetone* which contains not more than 0.3 per cent w/w of *water*, as determined by the following method.

WATER — Dilute about 12 ml of *acetyl chloride* to 100 ml with *toluene*. Transfer 10 ml to a dry, stoppered, 250 ml flask, preferably using a pipette with an automatic suction device, add 2 ml, accurately measured, of *pyridine*, immediately stopper tightly and shake vigorously avoiding wetting the stopper. Add 50 ml, accurately measured, of the reagent under examination and shake vigorously, avoiding wetting the stopper. Allow to stand for 5 minutes at room temperature, add by pipette 1.5 ml of *ethanol*, shake vigorously and allow to stand for 10 minutes. Add 25 ml of *ethanol* and titrate with *1 M sodium hydroxide* using *1-naphtholbenzein solution* as indicator. Add a slight excess of *1 M sodium hydroxide* and titrate with *1 M hydrochloric acid* after adding a few more drops of the indicator. Perform a blank determination. The difference between the two titrations represents the amount of sodium hydroxide equivalent to the *water* present.

1 ml of *1 M sodium hydroxide* is equivalent to 0.01802 g of H_2O.

Acetonitrile: Methyl Cyanide; $CH_3CN = 41.05$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 81°; wt. per ml, about 0.78 g.

Acetonitrile intended for use in spectrophotometry complies with the following test.

TRANS MITTANCE — Not less than 98 per cent in the range 230 to 420 nm using *water* as the blank.

Acetyl Chloride: $CH_3COCl = 78.50$

Analytical reagent grade of commerce.

Clear, colourless or very slightly yellow volatile liquid; smell, acrid or pungent; bp, about 51°; wt. per ml, about 1.1 g.

Acetyltyrosine Ethyl Ester; Ethyl *N*-acetyl-L-tyrosinate: $C_{13}H_{17}NO_4,H_2O = 269.3$

Analytical reagent grade of commerce.

A white, crystalline powder.

Acid Blue 83: $C_{45}H_{44}N_3NaO_7S_2 = 826.0$

Brown powder insoluble in cold *water*, slightly soluble in boiling *water* and in *ethanol*, soluble in *sulphuric acid*, *glacial acetic acid* and in dilute solution of alkali hydroxides.

Acid Blue 92; Coomassie Blue; Trisodium 8-hydroxy-4'-phenylaminoazonaphthalene-3,5',6-trisulphonate: $C_{26}H_{16}N_3Na_3O_{10}S_3 = 696$

General reagent grade of commerce.

Dark blue crystals.

Acid Blue 92 Solution; Coomassie Blue Solution: Dissolve 0.5 g of *acid blue 92* in a mixture of 10 ml of *glacial acetic acid*, 45 ml of *ethanol (95 per cent)* and 45 ml of *water*.

Acrylamide. $C_3H_5NO = 71.1$

Colourless or white flakes or a white or almost white, crystalline powder, very soluble in *water* and in *methanol*, freely soluble in *ethanol*.

mp. about 84°.

Acrylamide/bisacrylamide solution: Prepare a solution containing 292 g of *acrylamide* and 8 g of *methylenebisacrylamide* per litre of *water*.

Adrenaline: Of the Indian Pharmacopoeia.

Adrenaline Bitartrate: Of the Indian Pharmacopoeia.

Adrenaline Bitartrate, Noradrenaline-free; Adrenaline Acid Tartrate, Noradrenaline-free: Adrenaline bitartrate which complies with the following additional test.

NORADRENALINE — Determine the paper chromatography (2.4.15).

Mobile phase. Using in the bottom of the tank the lower layer obtained by shaking together 4 volumes of *1-butanol*, 1 volume of *glacial acetic acid* and 5 volumes of *water* and allowing to separate and use the upper layer.

Test solution. A 0.5 per cent w/v solution of the reagent under examination.

Apply to the paper 20 µl of the reagent. Develop for 24 hours and spray the dried paper with a freshly prepared 0.44 per cent w/v solution of *potassium ferricyanide* in *alkaline borate buffer pH 8.0*; the paper shows only one spot, which is pink.

Adrenalone Hydrochloride: 1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanone hydrochloride; 3',4'-Dihydroxy-2-(methylamino)acetophenone hydrochloride: $C_9H_{12}ClNO_3 = 217.7$

General laboratory reagent grade of commerce.

Agar: The dried extract from *Gelidium Sp.* and other algae belonging to the class Rhodophyceae.

Microbiological reagent grade of commerce.

Agarose for Electrophoresis: Electrophoretic reagent grade of commerce.

Neutral, linear polysaccharide, the main component of which is derived from agar.

β-Alanine: 3-Aminopropionic Acid; $C_3H_7NO_2 = 89.09$

General laboratory reagent grade of commerce.

Orthorhombic bipyramidal crystals; mp, about 200°, with decomposition.

Albumin, Bovine; Bovine Serum Albumin: Bovine serum albumin containing about 96.0 per cent of protein.

White to light brownish-yellow powder.

WATER (2.3.43) — Not more than 3.0 per cent w/w, determined on 0.8 g.

Aluminium: Al = 26.98

Analytical reagent grade of commerce.

A white, malleable, flexible, bluish metal available as bars, sheets, powder, strips or wire. In moist air an oxide film forms which protects the metal from corrosion.

Aluminium Potassium Sulphate; Alum; Potash Alum; Aluminium Potassium Sulphate Dodecahydrate; $AlK(SO_4)_2,12H_2O = 474.40$.

Analytical reagent grade of commerce.

Alumina, Anhydrous; Aluminium Oxide, Anhydrous: $Al_2O_3 = 101.96$

Use a grade of commerce consisting of γ-Al_2O_3, dehydrated and activated by heat treatment. The particle size is such that is passes through a 150 µm *sieve* but is retained on a 75 µm *sieve*.

Alumina, Deactivated; Aluminium Oxide, Deactivated: To a suitable basic alumina add 1.5 to 2 per cent of *water*, mix well and allow to stand overnight in a stoppered bottle. The product complies with the following test.

Prepare a column (20 cm x 10 mm) using the alumina and *hexane*. Add a solution of 0.25 g of *ergocalciferol* in 10 ml of *hexane*. When the level of the solution falls just to the top of the column, begin eluting with a 17.5 per cent v/v solution of *ether* in *hexane* adjusting the rate of flow, if necessary, to between 1 and 2 ml per minute. Collect 200 ml of the eluate; no calciferol is present. Collect a further 100 ml of eluate, it contains not less than 95 per cent of the calciferol used in the test, when determined by the Assay described under Calciferol Oral Solution.

Aluminium Chloride, Anhydrous: $AlCl_3 = 133.34$

General laboratory reagent grade of commerce.

A white, grey or yellow, crystalline powder, or crystalline masses, fuming in air, with a strong odour of hydrochloric acid.

Aluminium Chloride Solution: Dissolve 35.9 g of *anhydrous aluminium chloride* is sufficient *water* to produce 100 ml, add 0.5 g, of *activated charcoal*, stir for 10 minutes, filter and add to the filtrate with continuous stirring sufficient of a 10 per cent w/v solution of *sodium hydroxide* to adjust the pH to about 1.5.

CAUTION — Care should be taken in dissolving anhydrous aluminium chloride in water.

Aluminium Nitrate: $Al(NO_3)_3, 9H_2O = 375.13$

Analytical reagent grade of commerce.

Deliquescent crystals.

Aluminium Oxide G: Fine, white, homogeneous powder, of an average particle size between 10 µm and 40 µm containing about 10 per cent w/w of *calcium sulphate hemihydrate*. Complies with the following tests.

CALCIUM SULPHATE CONTENT — Weigh accurately about 0.25 g, add 3 ml of *2 M hydrochloric acid* and 100 ml of *water* and shake vigorously for 30 minutes. Filter and wash the residue with *water*. Titrate the combined filtrate and washings with *0.05 M disodium edetate* to within a few ml of the expected end-point. Add 4 ml of *10 M sodium hydroxide* and 0.1 g of *calcon mixture* and continue the titration until the colour changes from pink to full blue.

1 ml of *0.05 M disodium edetate* is equivalent to 0.00726 g of $CaSO_4, \frac{1}{2}H_2O$.

pH — About 7.5, determined in a suspension prepared by shaking 1 g with 10 ml of *carbon dioxide-free water*.

Aluminium Sulphate: $Al_2(SO_4)_3,16H_2O = 630.38$

Analytical reagent grade of commerce.

4-Aminobenzoic Acid; *p*-Aminobenzoic Acid: $NH_2C_6H_4COOH = 137.14$

General laboratory reagent grade of commerce.

A white or off-white crystals; mp, about 188°.

Complies with the following test.

HOMOGENITY — Carry out the test for Related substances described under Procaine Hydrochloride; the chromatogram shows only one spot.

4-Aminobenzoic Acid Solution. Dissolve 1.0 g of *4-aminobenzoic acid* in a mixture of 18 ml of *anhydrous acetic acid*, 20 ml of *water* and 1 ml of *phosphoric acid*. Immediately before use, mix 2 volumes of the solution with 3 volumes of *acetone*.

***N*-(4-Aminobenzoyl)-L-glutamic Acid**: $C_{12}H_{14}N_2O_5 = 266.26$

General laboratory reagent grade of commerce.

mp, about 173°.

4-Amino-butyric Acid; 4-Aminobutanoic acid; gamma amino butyric acid; GABA: $C_4H_9NO_2 = 103.1$

General reagent grade of commerce containing not less than 97 per cent of $C_4H_9NO_2$.

2-Amino-5-chlorobenzophenone: $C_{13}H_{10}ClNO = 231.68$

General laboratory reagent grade of commerce.

A yellow, crystalline powder; mp, about 99°.

Complies with the following test.

HOMOGENEITY — Carry out the test for Related substances described under Chlordiazepoxide Hydrochloride applying to the plate 5µl of a 0.05 per cent w/v solution in *methanol*; the chromatogram shows only one spot with an Rf value of about 0.9.

Store protected from light.

7-Aminodesacetoxycephasporanic Acid: $C_8H_{10}N_2O_3S = 214.20$

General laboratory reagent grade of commerce.

A light yellow powder.

4-Amino-3-hydroxynaphthalene-1-sulphonic Acid; Amino-2-naphthol-4-sulphonic Acid: $C_{10}H_9NO_4S = 239.25$

General laboratory reagent grade of commerce.

A crystalline powder; mp, about 300°, with decomposition.

Store protected from light.

Aminohydroxynaphthalenesulphonic Acid Solution: Dissolve 0.25 g of *4-amino-3-hydroxynaphthalene-1-sulphonic acid* in about 75 ml of a 15 per cent w/v solution of *sodium metabisulphite*, warming to assist solution if necessary. Add 25 ml of a 20 per cent w/v solution of *sodium sulphite*, mix and add sufficient of the *sodium metabisulphite solution* to produce 100 ml.

3-Aminomethylalizarin-*N,N*-diacetic Acid; Aminomethylalizarindiacetic acid; alizarin complexone dihydrate; $C_{19}H_{15}NO_8,2H_2O = 421.4$

A fine, ochre to orange-brown powder; melting point, about 185°.

Complies with the following test.

LOSS ON DRYING (2.4.19) — Not more than 10.0 per cent, determined on 1 g.

Aminomethylalizarindiacetic Acid Reagent

SOLUTION I — Dissolve 0.36 g of *cerium(III) nitrate* in sufficient *water* to produce 50.0 ml.

SOLUTION II — Suspend 0.7 g of *3-aminomethylalizarin-N,N- diacetic acid* in 50 ml of *water*. Dissolve with the aid of about 0.25 ml of *13.5M ammonia*, add 0.25 ml of *glacial acetic acid* and dilute to 100.0 ml with *water*.

SOLUTION III — Dissolve 6 g of *sodium acetate* in 50 ml of *water*, add 11.5 ml of *glacial acetic acid* and dilute to 100.0 ml with *water*.

To 33 ml of *acetone* add 6.8 ml of solution III, 1.0 ml of solution II and 1.0 ml of solution I and dilute to 50.0 ml with *water*. Use within 5 days.

Complies with the following test.

SENSITIVITY — To 1.0 ml of *fluoride standard solution (10 ppm F)* add 19.0 ml of *water* and 5.0 ml of the reagent under examination. After 20 minutes, a distinct blue colour is produced.

4-Aminomethylbenzoic Acid: $C_8H_9NO_2 = 151.2$

General laboratory reagent grade of commerce.

8-aminonaphthalene-2-sulphonic acid. $C_{10}H_9NO_3S$

Analytical reagent grade of commerce.

Aminonaphtholsulphonic Acid: $C_{10}H_9NO_4S = 239.3$

Analytical reagent grade of commerce.

Aminonaphthalenesulphonic Acid Solution. Mix in the following order 0.5 g of *8-aminonaphthalene-2-sulphonic acid*, 30 ml of *glacial acetic acid* and 120 ml of *water* and heat with stirring until dissolved. Allow to cool and filter. Use the solution within 3 weeks.

4-Aminophenazone; Aminopyrazolone; 4-Aminoantipyrine; 4-Amino-2, 3-dimethyl-1-phenyl-5-pyrazolone: $C_{11}H_{13}N_3O = 203.24$

General laboratory reagent grade of commerce.

A yellow to buff powder; mp, about 109°.

Aminophenazone Solution: A 0.1 per cent w/v solution of *4-aminophenazone* in *alkaline borate buffer pH 9.0*.

2-Aminophenol: $C_6H_7NO = 109.1$

Pale yellowish-brown crystals which rapidly become brown, sparingly soluble in *water*, soluble in *ethanol (95 per cent)*; mp, about 172°.

Store protected from light and moisture.

4-Aminophenol: *p*-Aminophenol; $NH_2C_6H_4OH = 109.13$

A white or almost white, crystalline powder; mp, about 186°.

3-Aminosalicylic Acid; 3-Amino-2-hydroxybenzoic acid: $C_7H_7NO_3 = 153$

General laboratory reagent grade of commerce.

4-Aminosalicylic Acid: 4-Amino-2-hydroxybenzoic acid; $C_7H_7NO_3 = 153$

General laboratory reagent grade of commerce.

Ammonia, x M: Solutions of any molarity x M may be prepared by diluting 75 x ml of *strong ammonia solution* to 1000 ml with *water*.

Ammonia-Ammonium Chloride Solution, Strong: Dissolve 67.5 g of *ammonium chloride* in 740 ml of *strong ammonia solution* and add sufficient *water* to produce 1000 ml.

Ammonia, Concentrated: Contains approximately 32.0 per cent w/w of *ammonia*, wt. per ml, about 0.883 to 0.889.

Store protected from atmospheric carbon dioxide at a temperature below 20°.

Ammonia-Cyanide Solution Sp.: Dissolve 2 g of *potassium cyanide* in 15 ml of *strong ammonia solution* and dilute to 100 ml with *water*.

Ammonia-Cyanide Wash Solution: To 35 ml of wash solution pH 2.5 add 4 ml of *ammonia-cyanide solution Sp.* and mix.

Ammonia, x M Ethanolic; Ammoniacal Ethanol, x M: Solutions of any morality x M may be prepared by diluting 75x ml of *strong ammonia solution* to 1000 ml with *methanol.*

Ammonia, x M Methanolic; Ammoniacal Methanol, x M: Solutions of any molarity x M may be prepared by diluting 75x ml of *strong ammonia solution* to 1000 ml with *methanol.*

Ammonia Solution, Dilute: Contains approximately 10 per cent w/w of NH_3. Dilute 425 ml of *strong ammonia solution* to 1000 ml.

Store protected from moisture in a cool place.

Ammonia, 18 M and 13.5 M: For *18M* and *13.5 M ammonia* use analytical reagent grade of commerce containing 35 per cent and 25 per cent w/v of ammonia and weighing 0.88 g and 0.91 g per ml, respectively. Solutions of any molarity x M may be prepared by diluting 75x ml of *13.5 M ammonia* or 56x ml of *18 M ammonia* to 1000 ml with *water.*

Ammonia Solution Sp.: *Strong ammonia solution* which complies with the following additional test.

Evaporate 10 ml to dryness on a water-bath. To the residue add 1 ml of *dilute hydrochloric acid Sp.* and evaporate to dryness. Dissolve the residue in 2 ml of *dilute acetic acid Sp.*, add sufficient *water* to produce 25 ml and add 10 ml of *hydrogen sulphide solution.* Any darkening produced is not greater than that of a blank solution containing 2 ml of *dilute acetic acid Sp.*, 2.0 ml of *standard lead solution (2 ppm Pb)* and sufficient *water* to produce 25 ml.

Ammonia Solution Sp., Dilute: *Dilute ammonia solution* which complies with the following test.

To 20 ml add 1 ml of *potassium cyanide solution Sp.*, dilute to 50 ml with *water* and add 0.1 ml of *sodium sulphide solution*; no darkening is produced.

Ammonia Solution, Iron-free: *Dilute ammonia solution* which complies with the following test.

Evaporate 5 ml nearly to dryness on a water-bath, add 40 ml of *water*, 2 ml of 20 per cent w/v solution of *iron-free citric acid* and 0.1 ml of *thioglycollic acid*, mix, make alkaline with *iron-free ammonia solution*, and dilute to 50 ml with *water*; no pink colour is produced.

Ammonia Solution, Strong; Ammonia: $NH_3 = 17.03$

Analytical reagent grade of commerce.

Contains 25.0 per cent w/w of NH_3 (limits, 24.5 to 25.5); wt. per ml, about 0.91 g; strength, about 13.5 M .

A clear, colourless liquid; odour, strongly pungent and characteristic.

Store protected from moisture in a cool place.

Ammonium Acetate: $CH_3COONH_4 = 77.08$

Analytical reagent grade of commerce.

A colourless crystals or crystalline masses; odour, slightly acetous; very deliquescent.

Ammonium Acetate, 0.1 M: Dissolve 7.71 g of *ammonium acetate* in 200 ml of *water*, add 1 ml of *glacial acetic acid* and dilute to 1000 ml with *water.*

Ammonium Acetate Solution: Dissolve 150 g of *ammonium acetate* in 200 ml of *water*, add 3 ml of *glacial acetic acid* and dilute to 1000 ml with *water.*

Use a freshly prepared solution.

Ammonium Carbonate: A variable mixture of ammonium bicarbonate (NH_4HCO_3) and ammonium carbamate (NH_2COONH_4). Contains the equivalent of not less than 30.0 per cent w/w of NH_3.

Analytical reagent grade of commerce.

Store protected from moisture at a temperature not exceeding 20°.

Ammonium Carbonate Solution. A 15.8 per cent w/v solution of *ammonium carbonate.*

Ammonium Carbonate, 2 M: Dissolve 192 g of *ammonium carbonate* in sufficient *water* to produce 1000 ml.

Ammonium Chloride: Of the Indian Pharmacopoeia.

Ammonium Chloride, 2 M: Dissolve 106.98 g of *ammonium chloride* in sufficient *water* to produce 1000 ml.

Ammonium Chloride-Ammonium Hydroxide Solution: Mix 1 volume of *water* and 2 volumes of *strong ammonia solution* and saturate with *ammonium chloride.*

Ammonium Chloride Solution: A 10 per cent w/v solution of *ammonium chloride.*

Ammonium Chloride Solution (Nessler's): Dissolve 3.15 g of *ammonium chloride* in sufficient quantity of ammonia-free *water* to produce 1000 ml.

Ammonium Chloride Solution, Dilute (Nessler's): Mix 10 ml of *ammonium chloride solution (Nessler's)* with sufficient quantity of *ammonia-free water* to produce 1000 ml.

Ammonium Citrate Solution: Dissolve, with cooling, 500 g of *citric acid* in a mixture of 200 ml of *water* and 200 ml of *strong ammonia solution*, filter and dilute to 1000 ml with *water.*

Ammonium Citrate Solution, Alkaline: Dissolve 9 g of *citric acid* in about 150 ml of *water* and add gradually 50 ml of *5 M ammonia.* Add 10 ml of *chloroform* and *dithizone solution* in 0.2 ml quantities until the *chloroform* layer, after vigorous shaking, is blue or purple. Discard the *chloroform* layer, add 10 ml of *chloroform* and 0.2 ml of *dithizone solution*, shake and allow to separate; the *chloroform* layer remains green. Discard the *chloroform* layer and wash the aqueous layer with successive quantities, each of 15 ml, of *chloroform* until

the washings are colourless. Dilute the aqueous layer to 300 ml of with *water*.

Ammonium Citrate Solution Sp.: Dissolve 40 g of *citric acid* in 90 ml of *water*, add 2 drops of *phenol red solution* and then add slowly *strong ammonia solution* until the solution acquires a reddish colour. Remove any lead present by extracting the solution with successive quantities, each of 30 ml, of *dithizone extraction solution* until the dithizone solution retains its orange-green colour.

Ammonium Dihydrogen Phosphate; Ammonium Dihydrogen Orthophosphate: Ammonium Phosphate, Monobasic: $NH_4H_2PO_4 = 115.03$

Analytical reagent grade of commerce.

Odourless crystals or crystalline powder.

Ammonium Formate: $C_2H_5NO_2 = 63.1$

General reagent grade of commerce.

mp, about 119° to 121°.

Store in an airtight container.

Ammonium Hexafluorogermanate (IV): $(NH_4)_2GeF_6 = 222.7$.
General reagent grade of commerce.

Ammonium Hexafluorophosphate: $NH_4PF_6 = 163.0$

General laboratory reagent grade of commerce

Ammonium Hydrogen Carbonate; Ammonium Bicarbonate: $NH_4HCO_3 = 79.1$

Content: minimum 99 per cent.

White or almost white, crystalline powder or colourless crystals, hygroscopic.

Ammonium Mercurithiocyanate Solution; Mercuric Ammonium Thiocyanate Solution: Dissolve 30 g of *ammonium thiocyanate* and 27 g of *mercuric chloride* in sufficient *water* to produce 1000 ml.

Ammonium Molybdate: $(NH_4)_6Mo_7O_{24},4H_2O$ = 1235.86
Analytical reagent grade of commerce.

A white crystals or crystalline masses, sometimes with a yellowish or greenish tint.

Ammonium Molybdate Reagent. Mix 10 ml of a 6.0 per cent w/v solution of *disodium arsenate*, 50 ml of *ammonium molybdate solution*, 90 ml of *1M sulphuric acid* and add sufficient *water* to produce 200 ml.

Condition the mixture at 37° for 24 hours and keep in amber flasks.

Ammonium Molybdate Solution: A 10 per cent w/v solution of *ammonium molybdate*.

Ammonium Molybdate Solution, Ethanolic

SOLUTION I — Dissolve 5 g of *ammonium molybdate* in 20 ml of *water* with the aid of heat.

SOLUTION II — Mix 150 ml of *ethanol (95 per cent)* with 150 ml of *water* and add, with cooling, 100 ml of *sulphuric acid*.

Add 80 volumes of solution II to 20 volumes of solution I immediately before use.

Ammonium Molybdate-Sulphuric Acid Solution: Dissolve 10 g of *ammonium molybdate* in sufficient *water* to produce 100 ml and add the solution slowly to 250 ml of cold *10 M sulphuric acid*.

Store in light-resistant plastic bottles.

Ammonium Nitrate: $NH_4NO_3 = 80.04$
Analytical reagent grade of commerce.

Ammonium Nitrate, 0.007 M: Dissolve 0.5603 g of *ammonium nitrate* in sufficient *water* to produce 1000 ml.

Ammonium Oxalate: $(COONH_4)_2,H_2O = 142.11$

Analytical reagent grade of commerce.

A colourless crystals.

Ammonium Oxalate, 0.1 M: Dissolve 14.21 g of *ammonium oxalate* in sufficient *water* to produce 1000 ml.

Ammonium Oxalate Solution: A 4.0 per cent w/v solution of *ammonium oxalate*.

Ammonium Persulphate; Ammonium Peroxodisulphate: $(NH_4)_2S_2O_8 = 228.20$

Analytical reagent grade of commerce.

A white, granular crystals or crystalline powder.

Ammonium Phosphate, Dibasic; Ammonium Phosphate; Diammonium Hydrogen Phosphate; Diammonium Orthophosphate; Diammonium Hydrogen Orthophosphate: $(NH_4)_2HPO_4 = 132.06$

Analytical reagent grade of commerce.

A odourless crystals or crystalline powder; taste, saline and cooling; gradually loses about 8 per cent NH_3, more on exposure to air.

Ammonium Phosphate, Dibasic, 0.2 M: Dissolve 26.41 g of *dibasic ammonium phosphate* in sufficient *water* to produce 1000 ml.

Ammonium Polysulphide Solution: Dissolve a sufficient quantity of *precipitated sulphur* in *ammonium sulphide solution* to produce a deep orange solution.

Ammonium Pyrrolidinedithiocarbamate; Ammonium Tetramethylenedithiocarbamate: $C_5H_{12}N_2S_2 = 164.28$

General Laboratory reagent grade of commerce.

Store in bottles containing a piece of *ammonium carbonate* in a muslin bag.

Ammonium Pyrrolidinedithiocarbamate Solution: A 1.0 per cent w/v solution of *ammonium pyrrolidinedithiocarbamate* that has been washed immediately before use with three quantities, each of 25 ml, of *4- methylpentan-2-one*.

Ammonium Reineckate; Ammonium Tetrathiocyanatodiaminochromate(III) Monohydrate: $NH_4[Cr(NH_3)_2(CNS)_4].H_2O$ = 354.42

General laboratory reagent grade of commerce.

A red powder or crystals.

Ammonium Reineckate Solution: A 1 per cent w/v solution of *ammonium reineckate*.

Use within a day of preparation.

Ammonium Sulphamate: $NH_2SO_3NH_4$ = 114.12

General laboratory reagent grade of commerce.

A white, crystalline powder or colourless crystals; mp, about 130°.

Ammonium Sulphate: $(NH_4)_2SO_4$=132.13

Analytical reagent grade of commerce.

A colurless crystals or white granules.

Ammonium Sulphide Solution: Saturate 120 ml of *6 M ammonia* with *hydrogen sulphide* and add 80 ml of *6 M ammonia*.

The solution should be freshly prepared.

Ammonium Thiocyanate: NH_4SCN = 76.12

Analytical reagent grade of commerce.

A colourless crystals.

Ammonium Thiocyanate, x M: Solutions of any molarity x M may be prepared by dissolving 76.12x g in sufficient *water* to produce 1000 ml.

Ammonium Thiocyanate Solution: A 10 per cent w/v solution of *ammonium Thiocyanate*.

Ammonium Thioglycollate Solution; Ammonium Mercaptoacetate solution: Add 300 ml of *water* to 50 ml of *thioglycollic acid*, neutralise with about 40 ml of *strong ammonia solution* and dilute with *water* to produce 500 ml.

Store protected from moisture.

Ammonium Vanadate; Ammonium Metavanadate: NH_4VO_3 = 116.98

Analytical reagent grade of commerce.

A white or slightly yellow, crystalline powder.

Amyl Acetate: $C_7H_{14}O_2$ = 130.19

Consists principally of 3-methylbutyl acetate with a small proportion of 2-methylbutyl acetate.

Analytical reagent grade of commerce.

A colourless liquid with a sharp, fruity odour; bp, about 140°; wt. per ml, about 0.87 g.

Amyl Alcohol; Isoamyl Alcohol: $C_5H_{12}O$ = 88.15

Consists principally of 3-methyl-*1-butanol* with a small proportion of 2-methyl-*1-butanol*.

Analytical reagent grade of commerce.

A colourless liquid; bp, about 130°; wt.per ml, about 0.81 g.

Aniline: $C_6H_5NH_2$ = 93.13

Analytical reagent grade of commerce

A colourless to pale yellow oily liquid; bp, about 184°; wt. per ml, about 1.02 g.

Store protected from light.

Aniline Hydrochloride; Benzenamine hydrochloride: C_6H_7N,HCl = 129.6

General laboratory reagent grade of commerce.

Crystals. It darkens on exposure to air and light, mp, about 198°.

Anion Exchange Resin, Strongly Basic: A gel-type resin in hydroxide form containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$, type 1] attached to a polymer lattice consisting of polystyrene cross- linked with 8 per cent of divinylbenzene.

Brown, transparent beads containing about 50 per cent of *water*; particle size, 0.2 to 10 mm; total exchange capacity, at least 1.2 milliequivalents per ml.

Anisaldehyde; 4-Methoxybenzaldehyde: $CH_3OC_6H_4CHO$ = 136.14

General laboratory reagent grade of commerce.

A colourless to pale yellow, oily liquid; odour, aromatic; bp, about 248°; wt. per ml, about 1.125 g.

Anisaldehyde intended for use in gas chromatography complies with the following additional test.

ASSAY — Determine the gas chromatography (2.4.13); the area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Anisaldehyde Solution; Anisaldehyde-Sulphuric Acid Reagent: Mix in the following order 0.5 ml of *anisaldehyde* 10 ml of *glacial acetic acid*, 85 ml of *methanol* and 5 ml of *sulphuric acid*.

Anisaldehyde Solution, Ethanolic: Mix in the following order 10 ml of *anisaldehyde*, 90 ml of *ethanol (95 per cent)* and 10 ml of *sulphuric acid*.

Anisaldehyde Solution, Methanolic: Slowly add 10.0 ml of *glacial acetic acid* and 5.0 ml of *sulphuric acid* to 55.0 ml of

methanol and cool to room temperature. Separately add 0.5 ml of *anisaldehyde* to 30.0 ml of *methanol*. Mix the two solutions thoroughly.

Store protected from light.

NOTE—Anisaldehyde solution, Methanolic should be freshly prepared.

Anthracene: $C_{14}H_{10}$=178.23

General laboratory reagent grade of commerce.

A white, crystalline powder; mp, about 218°.

Anthrone: $C_{14}H_{10}O$ = 194.23

Analytical reagent grade of commerce.

A pale yellow, crystalline powder; mp, about 155°.

Complies with the following test.

SENSITIVITY TO DEXTROSE — Add carefully 6 ml of a 0.2 per cent w/v solution in a mixture of 19 ml of *sulphuric acid* and 1 ml of *water* to 3 ml of *water* containing 15 mg of *anhydrous dextrose*. Heat in a water-bath for 5 minutes taking precautions against loss of *water* by evaporation. The solution is darker green than a solution prepared in a similar manner but omitting the dextrose.

Antimony Trichloride: $SbCl_3$ = 228.11

Analytical reagent grade of commerce.

A colourless crystals or flakes, fuming in moist air.

Store protected from light.

Antimony Trichloride Reagent

SOLUTION I — Dissolve 110 g of *antimony trichloride* in 400 ml of *1,2-dichloroethane*. Add 2 g of *anhydrous alumina*, mix and filter through sintered glass into a 500-ml volumetric flask. Dilute to 500 ml with *1,2-dichloroethane* and mix. Absorbance of the resulting solution measured in a 2-cm cell at about 500 nm, not more than 0.07 using *1,2-dichloroethane* as the blank (2.4.7).

SOLUTION II — Mix 100 ml of colourless, distilled *acetyl chloride* and 400 ml of *1,2-dichloroethane* and store in a cool place.

Mix 90 ml of solution I and 10 ml of Solution II.

Store in amber glass-stoppered bottles and use within 7 days. Discard any reagent in which colour develops.

Antimony Trichloride Solution: To 100 ml of a 22.0 per cent w/v solution of *antimony trichloride* in *ethanol-free chloroform* add 2.5 ml of *acetyl chloride* and allow to stand for 24 hours before use.

Aprotinin: General Laboratory reagent grade of commerce containing 10 to 20 trypsin inhibitor units per mg.

Arachidic Alcohol; Eicosan-1-ol: $C_{20}H_{42}O$ = 298.55

Purified reagent grade of commerce usually containing not less than 95 per cent of $C_{20}H_{42}O$.

Colourless, waxy or crystalline solid.

Arachis Oil: Of the Indian Pharmacopoeia.

Arginine: Of the Indian Pharmacopoeia.

Arsenic Trioxide: As_2O_3 = 197.82

Analytical reagent grade of commerce.

White or transparent, glassy amorphous lumps or crystalline powder.

Ascorbic Acid: Of the Indian Pharmacopoeia.

Atropine Sulphate: Of the Indian Pharmacopoeia.

Azomethine H Solution. Dissolve 0.45 g of *azomethine H* and 1 g of *ascorbic acid* in *water* with the aid of gentle heat and add sufficient *water* to produce 100 ml.

Barbaloin; 1,8-Dihydroxy-3hydroxymethyl-10-(β-D-gluco-pyranosyl)anthrone: $C_{21}H_{22}O_9,H_2O$ = 436.41

General laboratory reagent grade of commerce.

Lemon Yellow to dark yellow needles or crystalline powder, darkening on exposure to air and light; mp, about 148°.

Complies with the following test.

HOMOGENITY — Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 100 volumes of *ethyl acetate*, 17 volumes of *methanol* and 13 volumes of *water*.

Test solution. Dissolve 20 mg in 10 ml of *ethanol (70 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm. After development, dry the plate in air for 5 minutes and spray with a 5 per cent w/v solution of *potassium hydroxide* in *ethanol (50 per cent)*, heat at 105° for 15 minutes. The principal spot in the chromatogram obtained shows only one major reddish brown spot due to barbaloin (Rf value, 0.4 to 0.5).

Barbitone; Barbital; 5,5-Diethylbarbituric Acid: $C_8H_{12}N_2O_3$ = 184.19

General laboratory reagent grade of commerce.

Mp, about 190°.

Barbitone Sodium: $C_8H_{11}N_2NaO_3$ = 206.19

General laboratory reagent grade of commerce.

A white crystalline powder.

Barium Chloride: $BaCl_2,2H_2O$ = 244.27

Analytical reagent grade of commerce

Colourless crystals.

Barium Chloride Solution: A 10.0 per cent w/v solution of *barium chloride*.

Barium Hydroxide: $Ba(OH)_2, 8H_2O = 315.47$

Analytical reagent grade of commerce

Transparent crystals or white masses; mp, about 78°.

Barium Hydroxide, 0.1 M: Dissolve 31.547 g of *barium hydroxide* in sufficient *water* to produce 1000 ml.

Barium Hydroxide Solution: A 3.0 per cent w/v solution of *barium hydroxide* in *water*.

Barium Perchlorate: $Ba(ClO_4)_2$=336.23.

General laboratory reagent grade of commerce.

White powder.

Barium Sulphate: Of the Indian Pharmacopoeia.

Benzaldehyde: $C_7H_6O = 106.12$.

Analytical reagent grade of commerce

Colourless to pale yellow, oily liquid with an odour of almonds, bp, about 178°; wt per ml, about 1.04 g.

Store protected from light.

Benzalkonium Chloride Solution: Of the Indian Pharmacopoeia.

Benzene: $C_6H_6 = 78.11$

Analytical reagent grade of commerce

Colourless, transparent liquid; flammable; bp, about 80°; wt. per ml about 0.88 g.

Benzethonium chloride; Benzyldimethyl-2-{2-[4-(1,1,3,3-tetramethylbutyl) phenoxy]ethylammonium Chloride Monohydrate: $C_{27}H_{42}ClNO_2,H_2O = 466.12$

General Laboratory reagent grade of commerce.

Fine, white powder or colourless crystals; mp, about 163°

Store protected from light.

Benzidine; (1,1'-Biphenyl)-4,4'-diamine: $C_{12}H_{12}N_2 = 184.23$

General Laboratory reagent grade of commerce.

Pale buff, crystalline powder; darkens on exposure to air and light; mp, about 128°(when anhydrous and rapidly heated).

Store protected from light and moisture.

Complies with the following tests.

ORGANIC IMPURITIES — Dissolve 0.1 g in 5 ml of *glacial acetic acid*; the solution is clear (2.4.1) and not more than faintly coloured; add 5 ml of a mixture of equal volumes of *hydrogen peroxide solution* and *water*; no darkening is produced.

SENSITIVITY — 2 ml of the solution from the test for ORGANIC IMPURITIES, add 1 ml of a freshly prepared 0.0001 per cent solution of blood in *water*; a blue or greenish blue colour is produced.

Benzoic Acid: Of the Indian Pharmacopoeia.

Benzophenone: $C_{13}H_{10}O = 182.21$

General laboratory reagent grade of commerce.

mp, about 49°.

Benzoylarginine Ethyl Ester Hydrochloride: $C_{15}H_{23}ClN_4O_3$ = 342.8

General laboratory reagent grade of commerce.

mp, about 129°, $[\alpha]_D^{20}$, about –18° to –15°, [1 per cent w/v solution], A(1 per cent, 1cm), 310 to 340 determined at 227 nm using a 0.001 per cent w/v solution.

Benzoyl Chloride: $C_6H_5COCl = 140.57$

Analytical reagent grade of commerce.

Colourless liquid with a pungent odour; fuming in moist air; bp, about 197°; wt. per ml, about 1.21 g.

Benzoyl Peroxide: $C_{14}H_{10}O_4 = 242.2$

General laboratory reagent grade of commerce.

White or almost white granules; mp, after drying, about 104°.

For safety benzoyl peroxide should be kept moistened with about 23 per cent w/w of *water*.

Benzyl Alcohol: $C_7H_8O = 108.1$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 204°; wt. per ml, about 1.05 g.

Benzyl Benzoate: Of the Indian Pharmacopoeia.

Benzylpenicillin Sodium: Of the Indian Pharmacopoeia.

Benzyl Butyl Phthalate: $C_{19}H_{20}O_4 = 312$

General laboratory reagent grade of commerce.

Bismuth Subnitrate: Bismuth Oxynitrate; Bismuth Oxide Nitrate: $BiNO_4 = 286.98$

General laboratory reagent grade of commerce

White, microcrystalline powder; a basic salt containing about 80 per cent of Bi_2O_3.

Bis(trimethylsilyl)acetamide; N,O-Bis(trimethylsilyl)-acetamide: $CH_3CON[Si(CH_3)_3]_2 = 203.43$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 72°; wt.per ml, about 0.83 g.

Biuret; Carbonylurea: $C_2H_5N_3O_2 = 103.09$

General laboratory reagent grade of commerce.

White, hygroscopic crystals; mp, 188°to 190°, with decomposition.

Blue Tetrazolium; Tetrazolium Blue; Blue Tetrazolium Salt; 3,3'-(3,3'-Diemthoxy-4,4'-biphenylylene)bis(2,5-diphenyl-2H-tetrazolium Chloride: $C_{40}H_{32}Cl_2N_8O_2 = 727.65$

General laboratory reagent grade of commerce

Lemon Yellow crystals; mp, about 245°, with decomposition.

Blue Tetrazolium Solution: Dissolve 50 mg of blue tetrazolium in 10 ml of *aldehyde-free ethanol.*

Blue Tetrazolium Solution, Alkaline: Immediately before use mix 1 volume of a 0.2 per cent w/v solution of *blue tetrazolium* in *methanol* with 3 volumes of a 12 per cent w/v solution of *sodium hydroxide* in *methanol.*

Borax; Sodium Tetraborate; Disodium Tetraborate $Na_2B_4O_7,10H_2O=381.37$

Analytical reagent grade of commerce.

Transparent, colourless crystals, or white, crystalline powder; odourless; taste, saline, and alkaline; effloresces in dry air; on ignition, loses all its *water* of crystallisation.

Store protected from moisture.

Borax, 0.2 M: Dissolve 76.28 g of borax in sufficient *water* to produce 1000 ml.

Boric Acid: Of the Indian Pharmacopoeia.

Boric Acid Solution: Dissolve 5 g of *boric acid* in a mixture of 20 ml of *water* and 20 ml of *ethanol (95 per cent)* and dilute to 250 ml with *ethanol (95 per cent).*

Boron Trifluoride Solution: General laboratory reagent grade of commerce containing about 14 per cent of BF_3 in *methanol.*

Bromelain: A glycoprotein that is highly active thiol proteinase. It is found in the leaves and stems of the pineapple plant.

Yellowish-white to tan powder.

ASSAY — Carry out the method for determination of bromelain activity in the term of Gelatin Digestion Unit (GDU)/g.

Water pH 4.5: Adjusted to pH 4.5 with *0.1M hydrochloric acid.*

Gelatin substrate: Dissolve 25 g of gelatin in 375 ml of hot *water*, bring to boil and cool to 45°, adjusted to pH 4.5 with *0.1M hydrochloric acid* and dilute to 500.0 ml with *water* pH 4.5, Keep it at 45° (Use freshly prepared solution).

Buffer solution: Dissolve 150 g of *sodium chloride* in 700 ml of *water* pH 4.5, stir to dissolve, add 5.7 ml of *acetic acid*, adjusted to pH 4.5 with *sodium hydroxide* (50 per cent) and dilute 1000 ml with *water* pH 4.5.

Hydrogen peroxide solution (3 per cent): Dilute 2.5 ml of *hydrogen peroxide* to 25.0 ml with *water* pH 4.5.

Formaldehyde solution pH 9.0: Adjust 100 ml of *formaldehyde solution* to pH 9.0 with *0.1 M sodium hydroxide solution.*

Bromelain preparation: Weigh 100 mg of *bromelain* with a theoretical activity of 2400 GDU per gram. If the sample activity differs by more than 10 per cent from 2400 GDU per gram, determine the sample weight as follows:

$$\text{mg of sample} = \frac{2400 \times 100}{\text{theoretical activity}}$$

Transfer the sample to a 50-ml volumetric flask. Add 8.3 ml of buffer solution; stand for 30 minutes at room temperature and dilute to volume with *water* pH 4.5, stir for 10-15 minutes.

Procedure

Transfer 25 ml of Gelatin substrate to each of two 100-ml beakers containing stir bars and place them in a water-bath at 45° for 5 minutes. One beaker is for the test solution and the other for the blank solution.

NOTE — When adjusting the pH 6.0 to be cautious at pH 5.8; the pH increases slowly but minute additions of sodium hydroxide at this point will significantly increase the pH.

Test solution. Add 1.0 ml of bromelain preparation into the beaker and swirl. After exactly 20 minutes of incubation at 45°, add 0.1 ml of *hydrogen peroxide solution* (3 per cent) and swirl. Incubate for an additional 5 minutes. Remove the beaker from the water-bath and with constant stirring, adjusted to pH 6.0 with *0.1 M sodium hydroxide.* Add 10 ml of *formaldehyde solution* pH 9.0 with constant stirring. Titrate to a pH of 9.0 with *0.1 M sodium hydroxide.* Record the volume of titrant used. This is the test titer, T.

Blank solution. Run concurrently with the test solution by starting the blank solution determination 12 minutes after the test solution is started. Add 0.1 ml of *hydrogen peroxide solution* (3 per cent) to the blank solution and swirl. After exactly 20 minutes of incubation at 45 °, add 1.0 ml of bromelain preparation and swirl. Incubate for an additional 15 minutes. Remove the beaker from the water-bath and with constant stirring, adjusted to 6.0 with *0.1M sodium hydroxide.* Add 10 ml of *formaldehyde solution* pH 9.0 with constant stirring. Titrate to a pH of 9.0 with *0.1 M sodium hydroxide.* Record the volume of titrant used. This is the blank titer, B.

Calculation

1 Gelatin Digestion Unit (GDU) is the amount of enzyme, after 20 minutes of digestion at 45°, will liberate 1 mg of amino nitrogen from a standard gelatin solution at a pH of 4.5.

$$\frac{\text{GDU}}{\text{g}} = (\text{T} - \text{B}) \times 14 \times \text{M} \times \frac{50}{\text{W}}$$

where, T = ml of 0.1 M sodium hydroxide used with the test solution,

B = ml of 0.1 M sodium hydroxide used with the blank solution,

M = actual molarity of 0.1 M sodium hydroxide from standardization,

W = weight of bromelain taken (gram).

Bromine: Br_2 = 159.80

Analytical reagent grade of commerce.

Reddish brown, fuming, corrosive liquid ; wt. per ml, about 3.12 g.

Bromine, 0.0167 M: Dissolve 1 g of *potassium bromate* and 5 g of *potassium bromide* in sufficient *water* to produce 1000 ml.

Bromine Solution: Dissolve 9.6 ml of *bromine* and 30 g of *potassium bromide* in sufficient *water* to produce 100 ml.

Bromine Solution, Acetic: Dissolve 100 g of *potassium acetate* in *glacial acetic acid* and add 4 ml of *bromine* and sufficient *glacial acetic acid* to produce 1000 ml.

Bromine Water: Freshly prepared saturated solution obtained by shaking occasionally during 24 hours 3 ml of *bromine* with 100 ml of *water* and allowing to separate.

Store the solution over an excess of *bromine* in light resistant containers.

α-Bromo-2-acetonaphthone; Bromomethyl 2-Naphthyl Ketone: $C_{12}H_9BrO$ = 249.11.

Tannish pink crystals; mp, 81° to 83°.

4-Bromoaniline; *p*-Bromoaniline: C_6H_6BrN = 172.03

General reagent grade of commerce.

White to off-white crystals; mp, about 62°.

4-Bromoaniline Solution; Dissolve 2 g of *4-Bromoaniline* in 100 ml of *glacial acetic acid* saturated with *thiourea*.

Store protected from light. Prepare fresh weekly.

1-Butaneboronic Acid: $C_4H_{11}BO_2$ = 101.93

General Laboratory reagent grade of commerce.

Mp, about 95°.

1,2-Butanediol; 1-2-Butylene Glycol: $C_4H_{10}O_2$ = 90.12.

Viscous, colourless liquid; very hygroscopic; refractive index, between 1.4390 and 1.4410 at 20°.

Complies with the following additional test.

ASSAY — Determine by gas chromatography (2.4.13).

Chromatographic system

- a stainless steel column 1.8 m x 3 mm, packed with 20 per cent polyethylene glycol compound (average mol. wt. about 15,000) phase on support consisting of siliceous earth for gas chromatography, fluxcalcined by mixing diatomite with Na_2CO_3 flux and calcining above 900°, which is acid-washed, then water-washed until neutral, but not base-washed (The siliceous earth may be silanised by treating with an agent such as dimethyldichlorosilane to mask surface silanol groups)
- temperature: Injection port temperature maintained at about 265°, column temperature at about 150° and programmed to rise 8° per minute to about 210°,
- flame ionisaiton detector,
- carrier gas - helium.

The area of the butanediol peak is not less than 98 per cent of the total peak area.

1,3-Butanediol; 1,3-Butylene Glycol: $C_4H_{10}O_2$ = 90.12

Viscous, colorless liquid; very hygroscopic; Soluble in *water*, in *ethanol (95 per cent)*, in *acetone*, and in *methyl ethyl ketone*; practically insoluble in *aliphatic hydrocarbons*, in *benzene*, and in *toluene*. refractive index at 20; between 1.4390 and 1.4410.

ASSAY — Inject an appropriate specimen into a suitable gas chromatograph, equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 3 mm × 1.8 m stainless steel column containing 20 per cent phase G16 on support S1A; the injection port temperature is maintained at 265; the column temperature is maintained at 150 and programmed to rise 8 per minute to 210. The area of the butanediol peak is not less than 98 per cent of the total peak area.

1-Butanol; Butan-1-ol; *n*-Butyl Alcohol: $CH_3CH_2CH_2CH_2OH$=74.12

Analytical reagent grade of commerce.

Colourless liquid; bp, about 117°; wt.per ml, about 0.81 g.

2-Butanol; Butan-2-ol; *sec*-Butyl Alcohol:

$CH_3CH_2CHOHCH_3$=74.12

Colourless liquid; bp, about 99°; wt.per ml, about 0.81 g.

2-Butanol Reagent: *2-butanol* complying with the following tests.

DISTILLATION RANGE (2.4.8) — Not less than 95 per cent distils between 99° and 100°.

ASSAY — Not less than 99.0 per cent of $C_4H_{10}O$ determined by the method for Benzene and related substances described in the monograph of Isopropyl Alcohol.

2-Butanone: Butan-2-one; Methyl Ethyl Ketone: $C_2H_5COCH_3$ = 72.11

Chromatographic reagent grade of commerce.

Colourless, flammable liquid, odour, characteristic; bp, about 79°; wt. per ml, about 0.81 g.

Butyl Acetate: $CH_3COO(CH_2)_3CH_3 = 116.16$

Analytical reagent grade of commerce.

Clear, colourless, flammable liquid with a strong, fruity colour, bp, about 126°; wt. per ml. about 0.88 g.

***n*-Butylamine**; Butylamine: $C_4H_{11}N = 73.1$

General laboratory reagent grade of commerce.

A colourless liquid; bp, about 78°, refractive index at 20°, about 1.401.

Distill and use within 1 month.

Butylated Hydroxytoluene: Of the Indian Pharmacopoeia.

***n*-Butyl Chloride**; 1-Chlorobutane: $CH_3CH_2CH_2CH_2Cl = 92.57$

General laboratory reagent grade of commerce.

bp, about 78°; refractive index at 20°, about 1.402; wt. per ml about 0.886 g.

Butyl Paraben; Of the Indian Pharmacopoeia.

Butyric Acid; *n*-Butyric Acid; Butanoic Acid: $C_4H_8O_2 = 88.10$

General laboratory reagent grade of commerce.

Oily liquid; wt. per ml, about 0.96 g.

Cadmium Iodide: $CdI_2 = 366.23$

Analytical reagent grade of commerce.

Pearly white flakes or a crystalline powder.

Cadmium Iodide Solution: 5.0 per cent w/v solution of cadmium Iodide in *water*.

Cadmium Sulphate: $3CdSO_4,8H_2O = 769.52$

Analytical reagent grade of commerce.

Monoclinic crystals; odourless.

Caesium Chloride: $CsCl = 168.4$

Analytical reagent grade of commerce.

Calcium Acetate: $Ca(C_2H_3O_2)_2,H_2O = 176.18$

White, crystalline granules or powder.

Calcium Carbonate: Of the Indian Pharmacopoeia.

Calcium Chloride: Of the Indian Pharmacopoeia.

Calcium Chloride, x M: Solutions of any molarity xM may be prepared by dissolving 147x g of *calcium chloride* in sufficient *water* to produce 1000 ml.

Calcium Chloride, Anhydrous: $CaCl_2 = 110.99$

General laboratory reagent grade of commerce containing not less than 98.0 per cent w/w of $CaCl_2$, calculated with reference to the dried substance.

Dry white granules; very deliquescent.

Complies with the following test.

LOSS ON DRYING (2.4.19) — Not more than 5.0 per cent determined at 200°.

Calcium Chloride Solution: A 10.0 per cent w/v solution of *calcium chloride*.

Calcium Gluconate: Of the Indian Pharmacopoeia.

Calcium Oxide; Quicklime: $CaO = 56.08$

General laboratory reagent grade of commerce.

Dry, white lumps or powder; readily absorbs moisture and carbon dioxide from the atmosphere; when moistened with *water*, a reaction takes place with the evolution of heat and the lumps swell and fall to powder forming calcium hydroxide.

Calcium Sulphate; Calcium Sulphate Dihydrate; $CaSO_4,2H_2O = 172.17$

Analytical reagent grade of commerce.

White Powder, loses only part of water at 100° to 150°.

Calcium Sulphate Solution: A saturated solution of *calcium sulphate*.

Camphor: $C_{10}H_{16}O = 152.24$

Natural camphor or produced synthetically.

Mp, between 174 and 179°; $[\alpha]_D^{20}$, 41° to 43° (natural, 10 per cent w/v in *ethanol*); synthetic, optically inactive.

***dl*-10-Camphorsulphonic Acid**: $C_{10}H_{16}SO_4 = 232.29$

White to off-white crystals or powder; optically inactive; mp, about 199°.

Carbazole; Dibenzopyrolle: $C_{12}H_9N = 167.2$

Laboratory reagent grade of commerce.

mp, about 245°.

Carbomer; Carboxypolymethylene; Carbomer 934P: A synthetic high molecular weight polymer of acrylic acid cross linked with allylsucrose containing not less than 56.0 per cent and not more than 68.0 per cent of Carboxylic acid (–COOH) groups, calculated with reference to the substance dried at 80° at a pressure not exceeding 2.5 kPa for 1 hour.

White, fluffy powder; odour, slight and characteristic; hygroscopic.

Complies with the following tests.

ACIDITY — A 1 per cent w/v dispersion produces an orange colour with *thymol blue solution* and a yellow colour with *cresol red solution* (pH about 3).

VISCOSITY — Between 29400 and 39400 centipoises when determined by the following method.

Fix a stirrer capable of running at a speed of 1000 ± 10 rpm in a 1000 ml beaker containing 500 ml of *water* so that the shaft is

at an angle of 60°and to one side of the beaker and the propeller is near the bottom of the beaker. While stirring continously, add 2.5 g carefully and with uniform rate over a period of 45 to 90 seconds, ensuring that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 minutes. Remove the stirrer and place the beaker with its contents in a water-bath at 25° ± 0.2° for 30 minutes. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion and continue stirring at 300 ± 10 rpm. Titrate with a calomel-glass electrode system to a pH of 7.5 ± 0.3 by adding an 18 per cent w/v solution of *sodium hydroxide* below the surface, the end-point being determined potentiometrically (Approximately 6.2 ml of the *sodium hydroxide solution* will be required). Allow 2 to 3 minutes before final determination of pH. If the final pH exceeds 7.8, discard the mucilage and redetermine the pH by using lesser quantity of the *sodium hydroxide solution* for titration. Place this mucilage in the water-bath at 25° for 1 hour, and determine its viscosity by method C (2.4.28), without delay to avoid slight viscosity changes possible after 75 minutes of alkali addition. Equip a suitable rotating viscometer with a spindle having a cylinder of 1.47 cm diameter and 0.16 cm height attached to a shaft of 0.32 cm diameter, the distance from that of the cylinder to the lower tip of the shaft being 3.02 cm, and the immersion depth being 4.92 cm (No.6 spindle). Rotate the spindle at 20 rpm and record the scale reading on the viscometer. Calculate the viscosity in centipoises by multiplying the scale reading with spindle constant at 20 rpm.

Store protected from moisture.

Carbon Dioxide: CO_2 = 44.01

Cylinder of general laboratory grade of commerce.

Colourless, noncombustible gas; odourless.

Carbon Disulphide: CS_2 = 76.14

Analytical reagent grade of commerce.

Colourless, volatile flammable liquid with an unpleasant odour bp, about 46°; wt. per ml, about 1.26 g.

Carbon Tetrachloride; Tetrachloromethane: CCl_4 = 153.82

Analytical reagent grade of commerce.

Clear, colourless, volatile liquid; odour, characteristics; bp, about 76°; wt. per ml, about 1.59 g.

Carboxymethylcellulose; CMC: A monofunctional, weakly acidic, cation-exchange substance in a fibrous form. Stir 1 part into 15 parts of *0.5 M sodium hydroxide* and allow to stand for atleast 30 minutes. Remove the supernatant liquid and wash the residue with *water* until the washings have a pH 8. Stir the residue with 15 parts of *0.5 M hydrochloric acid* and allow to stand for 15 minutes. Repeat the acid treatment and finally wash with *water* until the washings are almost neutral. Suspend the residue in *water* containing 1 per cent v/v of *benzyl alcohol* and store until required.

Casein: Mixture of related phosphoproteins obtained from milk.

General laboratory reagent grade of commerce.

White, amorphous powder or granules.

Casein, Purified: White or slightly yellow, granular powder; odourless.

Casein, according Hammersten, which is commercially available may be used.

Casein Solution: Weigh accurately 4 g of *purified casein*, dissolve by shaking with 90 ml of *water*, adjust the pH to 7.0 and dilute with sufficient *water* to produce 100 ml.

Catechol; Pyrocatechol; Benzene-1,2-diol; *o*-Dihydroxybenzene: $C_6H_4(OH)_2$ = 110.11

General laboratory reagent grade of commerce.

White crystalline powder; mp about 103°.

Store protected from light.

Catechol Solution: A freshly prepared 10 per cent w/v solution of *catechol*.

Cation Exchange Resin: A resin in protonated form with sulphonic groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as beads and the particle size is specified after the name of the reagent in tests where it is used.

Casein Hydrolysate: A pancreatic digest of *casein* of general laboratory or microbiological grade of commerce.

Cellulose, Microcrystalline: Of the Indian Pharmacopoeia.

Cellulose F_{254}: A fine, white, homogeneous powder of an average particle size of less than 30 µm containing a fluorescent indicator with an optimal intensity at 254 nm.

Ceric Ammonium Nitrate; Ammonium Ceric Nitrate; Ammonium Cerium (IV) Nitrate: $[Ce(NO_3)_4,2NH_4NO_3$ = 548.23

Analytical reagent grade of commerce.

Ceric Ammonium Sulphate; Ammonium Ceric Sulphate; Ammonium Cerium (IV) Sulphate: $Ce(NH_4)_4(SO_4)_4,2H_2O$ = 632.53

Analytical reagent grade of commerce.

Yellow or yellowish orange crystals or a crystalline powder.

Cerium Sulphate; Ceric Sulphate: $Ce(SO_4)_2,4H_20$=404.3

Analytical reagent grade of commerce.

Yellow or orange yellow crystalline powder or crystals.

Cerous Nitrate; Cerium (III) Nitrate: $Ce(NO_3)_3\,6H_2O$ = 434.23.

General laboratory reagent grade of commerce.

Colourless or pale yellow, moist crystalline powder.

Cerous Nitrate Solution: Dissolve 0.22 g of *cerous nitrate* in 50 ml of *water*, add 0.1 ml of *nitric acid* and 50 mg of *hydroxylamine hydrochloride*, and dilute to 1000 ml with *water*.

Cetrimide: Of the Indian Pharmacopoeia.

Cetyl Palmitate; Hexadecyl Hexadecanoate; Hexadecyl Palmitate: $C_{32}H_{64}O_2 = 480.86$.

General chromatographic grade of commerce.

Cetyltrimethylammonium Bromide; *N*-hexadecyl-*N*,*N*,*N*-trimethylammonium bromides: $C_{19}H_{42}BrN = 364.5$

Analytical reagent grade of commerce.

A white, crystalline solid; mp, about 240°.

Charcoal, Activated: Of the Indian Pharmacopoeia.

Charcoal, Decolourising: Activated Charcoal of the Indian Pharmacopoeia which complies with the following additional test.

DECOLOURISING POWDER — Add 0.1 g to 50 ml of a 0.006 per cent w/v solution of *bromophenol blue* in *ethanol (20 per cent)* contained in a 250 ml flask, mix by rotating the vessel, allow to stand for 5 minutes and filter; the colour of the filtrate is not more intense than that of a solution prepared by diluting 1 ml of the *bromophenol blue solution* to 50 ml with *ethanol (20 per cent)*.

Chloral Hydrate: $C_2H_3Cl_3O_2 = 165.40$

General laboratory reagent grade of commerce.

Colourless, hygroscopic crystals with a sharp odour; mp, about 55°.

Complies with the following tests.

APPEARANCE OF SOLUTION — A 10.0 per cent w/v solution is clear (2.4.1) and colourless (2.4.1)

RESIDUE ON EVAPORATION — Not more than 0.2 per cent w/w, determined by evaporating 2 g to dryness on a water-bath.

Chloral Hydrate Solution: Dissolve 80 g of *chloral hydrate* in 20 ml of *water*.

Chloramine T; Chloramine; Sodium salt of *N*-chlorotoluene-p-sulphonamide: $C_7H_7ClNNaO_2S,3H_2O = 281.72$

General laboratory reagent grade of commerce.

White crystals or white crystalline powder; odour, that of chlorine; efflorescent; decomposed slowly by *ethanol*.

Chloramine T Solution; Chloramine Solution: A 2.0 per cent w/v solution of chloramine T.

Prepare immediately before use.

Chlorinated Lime; Bleaching Powder: Of the Indian Pharmacopoeia.

Chlorinated Lime Solution: Mix 100 g of chlorinated lime with 1000 ml of *water* in a stoppered bottle, set aside for 3 hours shaking occasionally and filter through calico.

The solution must be freshly prepared.

Chlorine: Greenish Yellow diatomic gas; odour, suffocating.

Chlorine Solution: A freshly prepared, saturated solution of chlorine in *water*.

4'-Chloroacetanilide: $CH_3CONHC_6H_4Cl = 169.61$

General laboratory reagent grade of commerce.

Colourless, rhombic crystals or plates; mp, about 178°.

4-Chloroaniline; *p*-Chloroaniline: $ClC_6H_4.NH_2 = 127.57$

General laboratory reagent grade of commerce.

White or faintly coloured crystals; mp about 71°.

4-Chlorobenzenesulphonamide: $C_6H_6ClNO_2S = 191.63$

General laboratory reagent grade of commerce.

mp, about 145°.

2-Chlorobenzoic Acid: $C_7H_5ClO_2 = 156.7$

General laboratory reagent grade of commerce.

bp, about 285°; mp, about 140°.

4-Chlorobenzoic Acid: $C_7H_5ClO_2 = 156.57$

General laboratory reagent grade of commerce.

Triclinic crystals; mp, about 240°.

4-Chloro-*o*-cresol; 4-Chloro-2-methylphenol: C_7H_7ClO = 142.56

General laboratory reagent grade of commerce.

mp, about 144°.

1-Chloro-2,4-dinitrobenzene: $ClC_6H_3(NO_2)_2 = 202.56$

Analytical reagent grade of commerce.

Pale Yellow crystals or crystalline powder; mp, about 51°.

2-Chloroethanol: Ethylene chlorohydrin; $C_2H_5ClO = 80.5$

Colourless liquid soluble in *ethanol (95 per cent)*, d_{20}^{20}, about 1.197; n_D^{20}, about 1.442; bp, about 130°; mp, about -89°.

Chloroform: Of the Indian Pharmacopoeia.

Chloroform, Ethanol-free; Chloroform, Alcohol-free: Wash repeatedly *chloroform* with *water*, dry with *anhydrous sodium sulphate* and distil; wt. per ml, about 1.489 g.

Ethanol-free chloroform must be freshly prepared.

CAUTION — Ethanol free chloroform is poisonous.

Chloroform IR: Spectroscopic reagent grade of commerce.

Chloroform, prepared: Distill *chloroform* in hard or borosilicate glass apparatus and collect the distillate in sufficient *ethanol* to make the final concentration of ethanol as 1 per cent v/v.

Chloroform Water: Shake 2.5 ml of *chloroform* with 900 ml of *water* until dissolved and dilute to 1000 ml with *water*.

5-Chloro-8-hydroxyquinoline; 5-Chloroquinolin-8-ol: $C_9H_6ClNO = 179.60$

General laboratory reagent grade of commerce.

mp, about 123°.

2-Chloronicotinic Acid: 2-Chloropyridine-3carboxylic acid; $C_6H_4ClNO_2 = 157.6$

White or almost white powder, mp, about 177°; content, minimum 95 per cent.

2-Chloro-4-nitroaniline: $C_6H_5ClN_2O_2 = 172.57$

General laboratory reagent grade of commerce.

Yellow to brown, crystalline powder; mp, about 107°.

Store protected from light.

Chloroplatinic Acid; Platinic Chloride: $H_2PtCl_6,6H_2O = 517.91$ or $H_2PtCl_6,3H_20 = 463.82$

General laboratory reagent grade of commerce.

Brownish, deliquescent, crystalline masses; contains not less than 37 per cent w/v of Pt.

ASSAY — Ignite 0.2 g to constant weight at 900°and weigh the residue (Platinum)

Store protected from light.

Chloroplatinic Acid Solution; Platinic Chloride Solution: A solution of *chloroplatinic acid* in *water* containing the equivalent of 5 per cent w/v of $H_2PtCl_6, 6H_2O$.

Cholesteryl Benzoate; 5-cholesten-3-yl-benzoate: $C_{34}H_{50}O_2 = 490.76$, mp, – 148-150°.

Analytical reagent grade of commerce.

5-Chlorosalicylic Acid: $C_7H_5ClO_3 = 172.57$

General laboratory reagent grade of commerce.

Choline Chloride; (2-Hydroxyethyl)trimethyl-ammonium chloride: $C_5H_{14}ClNO = 139.63$

General laboratory reagent grade of commerce.

Deliquescent crystals.

Complies with the following test.

HOMOGENEITY — Determine by thin-layer chromatography (2.4.17), coating the plate with microcrystalline cellulose.

Mobile phase. the upper layer obtained by shaking together for 10 minutes and then allowing to separate a mixture of 50 volumes of *1-butanol*, 40 volumes of *water* and 10 volumes of *anhydrous formic acid*.

Apply to the plate 5 µl of a 0.02 per cent w/v solution in *methanol*. The chromatogram obtained after drying the plate and spraying with *potassium iodobismuthate solution* shows only one spot.

Store protected from moisture.

Chromic Acid Solution: Dissolve 84 g of *chromium trioxide* in 700 ml of *water* and add slowly, with stirring, 400 ml of *sulphuric acid*.

Chromic-Sulphuric Acid; Chromic-Sulphuric Acid Mixture: A saturated solution of *chromium trioxide* in *sulphuric acid*.

Chromium Trioxide: $CrO_3 = 99.99$

Analytical reagent grade of commerce.

Dark red, deliquescent crystals or flakes or granular powder.

Store in a well-closed, glass-stoppered containers.

Chromogenic Substrate: *N*-Benzoyl-L phenylalanyl-L-valyl-L-arginine 4-nitroanilide Hydrochloride: $C_{33}H_{40}N_8O_6,HCl = 681.19$

General laboratory reagent grade of commerce.

Chromotropic Acid; 4,5-Dihydroxynaphthalene-2, 7-disulphonic Acid: $C_{10}H_8O_8S_2,2H_2O = 356.31$

General laboratory reagent grade of commerce.

White to brownish powder or white needles.

Chromotropic Acid Sodium Salt; Disodium 4,5-dihydroxynaphthalene-2,7-disulphonate: $C_{10}H_6Na_2O_8S_2,2H_2O = 400.30$

General laboratory reagent grade of commerce.

Pale brown powder or white needles or leaflets.

Complies with the following additional test.

SENSITIVITY TO FORMALDEHYDE — Dissolve 5 mg of *chromotropic acid* or *chromotropic acid sodium salt* in 10 ml of a mixture of 9 ml of *sulphuric acid* and 4 ml of *water*. Dilute exactly 0.5 ml of *formaldehyde solution* with *water* to make 1000 ml. Add 5 ml of this solution to the solution of chromotropic acid or of its sodium salt and heat in a water-bath for 30 minutes; a violet colour is produced. Use a solution prepared in the same manner using *water* in place of the formaldehyde solution for comparison.

Chromotropic Acid Solution; Chromotropic Acid-Sulphuric Acid Solution: Dissolve 50 mg of *chromotropic acid* or *chromotropic acid sodium salt* in 100 ml of a mixture of 9 ml of *sulphuric acid* and 4 ml of *water*.

Cineole; Eucalyptol; 1,8-Epoxy-p-menthane: $C_{10}H_{18}O = 154.25$

A grade of commerce specially supplied for o-cresol determinations.

Colourless liquid with a camphoraceous odour; bp, about 176°; refractive index at 20°, about 1.457; wt. per ml, about 0.92 g.

Cinnamaldehyde; Cinnamic Aldehyde: $C_9H_8O = 132.15$

General laboratory reagent grade of commerce.

Pale-yellow, oily liquid; wt.per ml, about 1.05 g.

Store protected from light in a cool place.

Citrate-Cyanide Wash Solution: To 50 ml of *water* add 50 ml of *ammonium citrate solution Sp.* and 4 ml of *potassium cyanide solutions Sp.*, mix and adjust the pH to 9.0, if necessary, with *strong ammonia solution.*

Citric Acid: Of the Indian Pharmacopoeia.

Citric Acid, 0.1 M: Dissolve 21.0 g of *citric acid* in sufficient *water* to produce 1000 ml.

Citric Acid, Anhydrous: Of the Indian Pharmacopoeia.

Citric Acid, Iron-Free: *Citric Acid* which complies with the following additional test.

Dissolve 5 g in 40 ml of *water*, add 2 drops of *thioglycollic acid*, mix, make alkaline with *iron-free ammonia solution*, and dilute to 50 ml with *water*; no pink colour is produced.

Citric- Molybdic Acid Solution: Mix 54 g of *molybdic oxide* with 200 ml of *water*, add 11 g of *sodium hydroxide* and heat, with stirring, until almost complete solution has been obtained. Dissolve 60 g of *citric acid* in 250 ml of *water* and add 140 ml of *hydrochloric acid.* Add the first solution to the second, stirring continuously, cool, filter if necessary, dilute to 1000 ml with *water* and add, dropwise, sufficient of a 1 per cent w/v solution of *potassium bromate* to discharge the green colour.

Store protected from light and moisture.

Clotting Factor V Solution: Clotting Factor V Solution may be prepared by the following method or by any other method that excludes factor VIII.

Prepare from fresh oxalated bovine plasma by fractionation at 4° with a saturated solution of *ammonium sulphate* prepared at 4°. Use the fraction precipitating between 38 and 50 per cent saturation (which contains clotting factor V not significantly contaminated with clotting factor VIII), dialysed to remove ammonium sulphate and diluted with *saline solution* to produce a solution containing 10 to 20 per cent of the amount of clotting factor V present in fresh normal human plasma.

Determine the clotting factor V content of the solution in the following manner. Prepare two dilutions in *imidazole buffer pH 7.4* to contain 1 volume to the solution under examination in 10 volumes and 20 volumes, respectively. Test each dilution as follows:

Mix 0.1 ml of each of *substrate plasma deficient in clotting factor V*, the dilution being tested, *thrombokinase extract* and *0.025 M calcium chloride*. Record as the clotting time the interval between the addition of the *calcium chloride solution* and the first indication of fibrin formation, which may be observed visually or by mechanical means.

Similarly determine the clotting times, in duplicate, for four dilutions of pooled norman human plasma in *imidazole buffer pH 7.4* containing 1 volume in 10 volumes (equivalent to 100 per cent of clotting factor V), in 50 volumes (20 per cent), in 100 volumes (10 per cent) and in 1000 volumes (1 per cent), respectively.

To calculate the result, plot the mean of the clotting times for each dilution of human plasma on double cycle log/log paper against the equivalent percentage for clotting actor V and read the percentage of clotting factor V for the two dilutions of *clotting factor V solution* by interpolation from the curve. The mean of the two results is taken as the percentage of clotting factor V in the solution.

Cobalt Acetate; Cobaltous Acetate; Cobalt (II) Acetate: $(CH_3COO)_2Co,4H_2O = 249.08$

General laboratory reagent grade of commerce.

Deep red crystals; odour, faintly acetous.

Cobalt Chloride; Cobaltous Chloride; Cobalt (II) Chloride: $CoCl_2,6H_2O = 237.93$

Analytical reagent grade of commerce.

Deep red crystals, slightly deliquescent.

Cobalt Chloride Solution: Dissolve 6.5 g of *cobalt chloride* in 8 ml of *2 M hydrochloric acid* and dilute to 100 ml with *water.*

Cobalt Nitrate; Cobaltous Nitrate; Cobalt (II) Nitrate: $Co(NO_3)_2,6H_2O = 291.03$

Analytical reagent grade of commerce.

Red Crystals; deliquescent.

Cobalt Thiocyanate Solution: Dissolve 6.8 g of *cobalt chloride* and 4.3 g of *ammonium thiocyanate* in sufficient *water* to produce 100 ml.

Codeine; (5*R*,6S)-4,5-Epoxy-3-methoxy-*N*-methyl-morphin-7-en-6-ol monohydrate: $C_{18}H_{21}NO_3H_2O = 317.37$

Colourless crystals or white crystalline powder; odourless; mp, about 157°; $[\alpha]_D^{20}$, about – 146° to – 142° [2 per cent w/v in *ethanol (95 per cent)*]; contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{18}H_{21}NO_3$, calculated with reference to the dried substance.

Store protected from light and moisture.

Codeine Phosphate: Of the Indian Pharmacopoeia.

Colchicine: Of the Indian Pharmacopoeia.

Coomassie staining solution. A 1.25 g per litre solution of *acid blue 83* in a mixture consisting of 1 volume of *glacial acetic acid*, 4 volume of *methanol* and 5 volume of *water*.

Copper; Copper Foil; Copper Turnings: Cu = 63.55

Pure metal known commercially under the term 'electrolytic'.

Usually in the form of foil, turnings or borings.

Copper Solution, Alkaline

SOLUTION I — Dissolve 8 g of *sodium hydroxide* in 200 ml of *distilled water*, add 40 g of *sodium carbonate* and make up the volume to 1000 ml with *water*.

SOLUTION II — Dissolve 4 g of *potassium tartrate* and 2 g of *cupric sulphate* in sufficient *water* to produce 100 ml.

Mix 50 ml of solution I and 1 ml of solution II just before use.

Cresol: Of the Indian Pharmacopoeia.

Cupric Acetate; Copper (II) Acetate: $(CH_3COO)_2Cu, H_2O$ = 199.65

Analytical reagent grade of commerce.

Dark green crystals.

Cupric Chloride; Copper (II) Chloride: $CuCl_2, 2H_2O$ = 170.48

Analytical reagent grade of commerce.

Green to blue powder or crystals, deliquescent in moist air, efflorescent in dry air.

Cupric Chloride-Pyridine Reagent; Copper Chloride-Pyridine Reagent: Dissolve 40 mg of *cupric chloride* in *pyridine*, warming until complete dissolution is effected, and cool. Add 1 ml of *carbon disulphide* and sufficient *pyridine* to produce 100 ml.

Cupri-Citric Solution; Copper-Citric Solution; Sodium Cupri-Citrate Solution: Dissolve 25 g of *cupric sulphate*, 50 g of *citric acid* and 144 g of *anhydrous sodium carbonate* in sufficient *water* to produce 1000 ml.

Cupric Sulphate; Copper(II) Sulphate: $CuSO_4, 5H_2O$ = 249.68

Analytical reagent grade of commerce.

Large, blue or ultramarine crystals or blue granules or light blue powder, slowly efflorescent in air.

Cupric Sulphate, 0.02 M: Dissolve 5.0 g of *cupric sulphate* in *water* and dilute to 1000 ml with *water*.

Cupric Sulphate Solution; Copper Sulphate Solution: A 12.5 per cent w/v solution of *cupric sulphate*.

Cupric Sulphate Solution, Weak; Cupric Sulphate Solution, Dilute: A 10.0 per cent w/v solution of *cupric sulphate*.

Cupric Sulphate with Pyridine Solution: Dissolve 4 g of *cupric sulphate* in 90 ml of *water* and add 30 ml of *pyridine*. The solution should be freshly prepared.

Cupri-Tartaric Solution; Modified Potassium Cupritartrate Solution.

SOLUTION I — Dissolve 34.6 g of *cupric sulphate* in sufficient *water* to produce 500 ml.

SOLUTION II — Dissolve 173 g of *sodium potassium tartrate* and 50 g of *sodium hydroxide* in 400 ml of *water*, heat to boiling, allow to cool and dilute to 500 ml with freshly boiled and cooled *water*.

Mix equal volumes of solution I and II immediately before use.

Cuprous Chloride; Copper (I) Chloride: CuCl = 99.00

Analytical reagent grade of commerce.

Curcumin; 1,7-Bis(4-hydroxy-3-methoxyphenyl)-hepta-1, 6-dien-3,5-dione: $C_{21}H_{20}O_6$ = 368.39

General laboratory reagent grade of commerce.

Orange brown, crystalline powder; mp, about 183°.

Cyanoacetic Acid; Malonic acid mononitrile: $C_3H_3NO_2$ = 85.1

General laboratory reagent grade of commerce.

mp, about 67°.

Store protected from moisture.

Cyanogen Bromide Solution: Add, drop-wise with cooling, *0.1 M ammonium thiocyanate* to *bromine water* until the colour disappears.

Prepare immediately before use.

β-Cyclodextrin. Of the Indian Pharmacopoeia.

Cyclohexane: C_6H_{12} = 84.16

Analytical reagent grade of commerce.

Clear, colourless liquid: bp, about 81°; wt. per ml, about 0.78 g.

Cyclohexane intended for use in spectrophotometry complies with the following test.

TRANSMITTANCE — Not less than 45 per cent at about 220 nm, 80 per cent at about 235 nm, 90 per cent at about 240 nm and 98 per cent at about 250 nm, using *water* as the blank.

Cyclohexane intended for use in fluorimetry complies with the following additional test.

FLUORESCENCE — Under radiation at about 365 nm, the *fluorescence* measured at about 460 nm is not more intense that that of a solution containing 0.002 mg per ml of *quinine* in *0.05 M sulphuric acid*.

3-Cyclohexylpropionic acid: $C_9H_{16}O_2$ = 156.2

Analytical reagent grade of commerce.

bp, about 30°; wt. per ml, about 0.99 g.

Cysteine Hydrochloride; α-Amino-β-mercaptopropionic acid hydrochloride: $[SHCH_2CH(NH_2)CO_2H],HCl = 157.66$

White crystals; mp, about 177°, with decomposition.

Cysteine Hydrochloride Solution: Dissolve 0.5 g of *cysteine hydrochloride*, accurately weighed, in 10 ml of *water* and adjust the pH to 7.0.

The solution should be freshly prepared.

1-Decanol; Decan-1-ol: $C_{10}H_{22}O = 158.28$

General laboratory reagent grade of commerce.

Moderately viscous liquid; wt. per ml, about 0.83 g.

Dequalinium Chloride: Of the Indian Pharmacopoeia.

Destaining solution. A mixture consisting of 1 volume of *glacial acetic acid*, 4 volume of *methanol* and 5 volume of *water*.

Deuterium Oxide: $D_2O = 20.03$

General laboratory reagent grade of commerce with a minimum isotopic purity of 99.7 per cent.

A colourless liquid; bp, about 101°; d_{20}^{20}, about 1.11; n_D^{20}, about 1.328

Dextrose, Anhydrous: Of the Indian Pharmacopoeia.

Diacetone Alcohol; 4-Hydroxy-4-methyl-3-pentanone: $C_6H_{12}O_2 = 116.16$

General laboratory reagent grade of commerce containing not less than 98.5 per cent of $C_6H_{12}O_2$, determined by gas chromatography (2.4.13).

Diammonium Hydrogen Citrate: $(NH_4)_2C_6H_6O_7 = 226.19$

General laboratory reagent grade of commerce.

2,3-diaminonapthalene: $C_{10}H_{10}N_2 = 158.2$

Analytical reagent grade of commerce.

Diatomaceous Support, Acid-washed, Silanised: Use a suitable laboratory reagent grade of commerce.

Acid-washed diatomaceous support that has been silanised with dimethyldichlorosilane or other suitable silanising agents.

Diazobenzenesulphonic Acid Solution: Heat 0.2 g of *sulphanilic acid* with 20 ml of *1 M hydrochloric acid* until dissolved, cool to about 4° and add, dropwise, 2.2 ml of a 4 per cent w/v solution of *sodium nitrite*, swirling continuously. Allow to stand in ice for 10 minutes and add 1 ml of a 5 per cent w/v solution of *sulphamic acid*.

Diazobenzenesulphonic Acid Solution, Dilute: Dissolve 0.9 g of *sulphanilic acid* in a mixture of 30 ml of *2 M hydrochloric acid* and 70 ml of *water*. To 3 ml of this solution add 3 ml of a 5 per cent w/v solution of *sodium nitrite*. Cool in ice for 5 minutes, add 12 ml of the *sodium nitrite* solution and cool again. Dilute the solution to 100 ml with *water* and keep the reagent in ice. Prepare extemporaneously but allow to stand for 15 minutes before use.

Dibenzosuberone; Dibenzo[*a*,*d*]cyclohepta-1,4-dien-3-one; 10,11-Dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5-one: $C_{15}H_{12}O = 208.26$

General laboratory reagent grade of commerce.

mp, about 24°.

Di-*n*-butylamine: $C_8H_{19}N = 129.25$

General laboratory reagent grade of commerce.

bp, about 159°; refractive index at 20°, about 1.417.

Dibutyl Ether: $C_8H_{18}O = 130.22$

General laboratory reagent grade of commerce.

Colourless, flammable liquid; bp, about 140°; wt. per ml. About 0.77 g.

Do not distill unless the dibutyl ether complies with the test for peroxides.

PEROXIDES — Place 8 ml of *starch iodide solution* in a 12 ml glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the reagent under examination, shake vigorously and allow to stand protected from light for 30 minutes; no colour is produced.

Dichloroacetic Acid: $C_2H_2Cl_2O_2 = 128.95$

General laboratory reagent grade of commerce.

bp, about 193°; refractive index at 20°, about 1.466; wt. per ml, about 1.563 g.

Dichloroacetic Acid Solution: Dilute 67 ml of *dichloroacetic acid* to 300 ml with *water* and neutralise to *litmus paper* using *10 M ammonia*. Cool, add 33 ml of *dichloroacetic acid* and dilute with *water* to 600 ml.

2,6-Dichloroaniline: $C_6H_5Cl_2N = 162.02$

Analytical reagent grade of commerce.

Off-white powder; mp, between 38° to 41°.

2,4-dichlorobenzoic Acid: $C_7H_4Cl_2O_2 = 191.0$

Faintly beige powder.

mp, about 160°

2, 3-dichloro-5,6-dicyanobenzoquinone: $C_5H_{10}O_2 = 102.13$

General laboratory reagent grade of commerce

1,2-Dichloroethane; Ethylene Chloride: $ClCH_2CH_2Cl = 98.96$

Analytical reagent grade of commerce.

Colourless liquid; odour, similar to that of chloroform; bp, about 83°; wt. per ml, about 1.25 g.

1,2-Dichloroethane, Purified: Purify *1,2-dichloroethane* by passing through a column of granular *silica gel* (20 to 200 mesh).

2,7-Dichlorofluorescein: $C_{20}H_{10}Cl_2O_5 = 401.19$

Adsorption indicator grade of commerce.

Yellowish brown to yellowish orange powder.

5,7-Dichloro-8-hydroxyquinoline;

5,7-Dichloroquinolin-8-ol: $C_9H_5Cl_2NO = 214.05$

General laboratory reagent grade of commerce.

Yellow-tan crystals; mp, about 181°.

Dichloromethane; Methylene Chloride: $CH_2Cl_2 = 84.93$

Analytical reagent grade of commerce.

Volatile, sweet-smelling liquid; bp, about 40; wt. per ml, about 1.32 g.

Dichloromethane intended for use in fluorimetry complies with the following additional test.

FLUORESCENCE — Under radiation at about 365 nm, the *fluorescence* measured at about 460 nm is not more intense than that of a solution containing 0.002 mg per ml of *quinine* in *0.05 M sulphuric acid*.

Dichloromethane IR: Spectroscopic reagent grade of commerce.

Dichloromethane Reagent: Add 50 g of *sodium hydrogen carbonate* to 1000 ml of *dichloromethane*, allow to stand overnight and filter.

2,6-Dichlorophenolindophenol Sodium; Tillman's Reagent; Sodium salt of 2,6-dichloro-*N*-(4-hydroxy-phenyl)-1,4-benzoquinone monoimine: $C_{12}H_6Cl_2NNaO_2,2H_2O = 326.11$

Analytical reagent grade of commerce.

Dark green powder.

2,6-Dichlorophenolindophenol Solution: Warm 0.1 g of 2,6-*dichlorophenolindophenol sodium* with 100 ml of *water* and filter.

Must not be used later than 3 days after preparation.

2,6-Dichlorophenolindophenol Standard Solution:

Dissolve 50 mg of *2,6-dichlorophenolindophenol sodium* in 100 ml of *water* and filter. Dissolve 20.0 mg of *L-ascorbic acid* in 10 ml of a freshly prepared 20 per cent w/v solution of *metaphosphoric acid* and dilute to 250 ml with *water*. Titrate 5.0 ml rapidly with the *2,6-dichlorophenolindophenol solution*, added from a microburette graduated in units of 0.01 ml, until the pink colour of the dye persists for 10 seconds, the titration taking not more than 2 minutes. Dilute the 2,6-dichlorophenolindophenol solution with *water* so that 1 ml of the solution is equivalent to 0.1 mg of ascorbic acid, $C_6H_8O_6$.

The solution must not be used later than 3 days after preparation and must be standardised immediately before use.

2,6-Dichloroquinone-4-chloromide; Dichloroquinone-chloroimine: $C_6H_2Cl_3NO = 210.45$

General laboratory reagent grade of commerce.

Yellow or orange crystalline powder; mp, about 66°.

Dicyandiamide: $C_2H_4N_4 = 84.08$

General laboratory reagent grade of commerce.

White, crystalline powder; mp, about 211°.

Di-2-cyanoethyl Ether; (2-Cyanoethyl)-ether: $C_{12}H_{23}N = 124.14$.

Chromatographic grade of commerce.

bp, about 111°; refractive index at 20°, 1.4400.

Dicyclohexylamine: *N,N*-Dicyclohexylamine: $C_{12}H_{23}N = 181.32$

General reagent grade of commerce.

Colourles liquid; refractive index at 20°, about 1.484; freezing point, 0° to 1°; boiling point, about 256°.

1,3-Dicyclohexylurea: $C_{13}H_{24}N_2O = 224.34$

General laboratory reagent grade of commerce.

mp, about 232°.

Diethanolamine: Of the Indian Pharmacopoeia.

2,5-Diethoxytetrahydrofuran: $C_8H_{16}O_3 = 160.21$

General laboratory reagent grade of commerce consisting of a mixture of *cis*- and *trans*- isomers.

Colourless or slightly yellow liquid; wt. per ml, about 0.97 g.

Diethylamine: $C_4H_{11}N = 73.14$

Analytical reagent grade of commerce.

A volatile, colourless liquid; bp, about 55°, relative density (2.4.29), about 0.71.

Diethylaminoethylcellulose: Chromatographic grade of commerce.

Diethylaminoethyldextran: Anion exchange resin presented as the hydrochloride.

Powder, forming gels with *water*.

***N,N*-Diethylaniline**: $C_{10}H_{15}N = 149.23$

General laboratory reagent grade of commerce.

Pale yellow liquid with an ammoniacal odour; bp, about 217°; wt. per ml, about 0.93 g.

Diethylene Glycol; Digol: $(CH_2OHCH_2)_2O = 106.12$

Analytical reagent grade of commerce.

Clear, colourless liquid; wt. per ml, about 1.12 g.

Diethylene Glycol Succinate Polyester: Gas chromatographic reagent grade of commerce.

***N,N*-Diethylethylenediamine**; *N,N*-Diethyl-1, 2-diamino-ethane: $C_6H_{16}N_2$= 116.21

General laboratory reagent grade of commerce.

Colourless or slightly yellow, slightly oily liquid with a strong odour of ammonia; bp, about 146°; wt. per ml, about 0.83 g.

Complies with the following additional test.

WATER (2.3.43) — Not more than 1.0 per cent determined on 0.5 g.

Diethylphenylenediamine sulphate; *N,N*-Diethyl-p-phenylenediamine sulphate,$C_{10}H_{16}N_2.H_2SO_4$ = 262.3

White or slightly yellow powder, soluble in *water*.

melting point, about 185°, with decomposition.

Store protected from light.

Digitoxin: Of the Indian Pharmacopoeia.

Digoxin Reagent: Add 98 ml of *glacial acetic acid* to 2 ml of *sulphuric acid* and add 0.1 ml of a 5 per cent w/v solution of *anhydrous ferric chloride* in *glacial acetic acid*.

2,5-Dihydroxybenzoic Acid; Gentisic acid: $C_7H_6O_4$ = 154.1

Light yellow crystals; mp, about 200°.

1,5-Di-iodopentane: $C_5H_{10}I_2$ = 323.94

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 101°.

5,7-Di-iodo-8-hydroxyquinoline;
5,7-Di-iodoquinolin-8-ol: $C_9H_5I_2NO$ = 396.95

General laboratory reagent grade of commerce.

mp, about 214°, with decomposition.

Di-isopropylamine: $[(CH_3)_2CH]_2NH$ = 101.19

Colourless liquid; refractive index at 20°, 1.3915 to 1.3935; contains not less than 98 per cent of $C_6H_{15}N$.

ASSAY — Determined by gas chromatography (2.4.13).

Chromatographic system
- a stainless steel column 1.8 m x 3.2 mm, packed with a cross-linked polystyrene support,
- temperature: column 250° and of the detector at 310°, the column temperature being programmed to rise at 10° per minute from 50° to 220°,
- flame ionisation detector,

Di-isopropyl Ether; Isopropyl Ether: $[(CH_3)_2CH]_2O$ = 102.18

General laboratory reagent grade of commerce.

Colourless liquid with a characteristic odour; bp, about 68°; wt. per ml, about 0.72 g.

NOTE — Do not distil unless the di-isopropylether complies with the test for peroxides.

PEROXIDES — Place 8 ml of *starch iodide solution* in a 12 ml glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the reagent under examination, shake vigorously and allow to stand protected from light for 30 minutes; no colour is produced.

Store protected from light. The name and concentration of any added stabiliser are stated on the label.

Di-isopropylethylamine; *N,N*-Diisopropylethylamine: $C_8H_{19}N$ = 129.24

General laboratory reagent grade of commerce.

Clear, colorless liquid; Soluble in *glacial acetic acid*; refractive index at 20; between 1.4125 and 1.4145.

ASSAY — Accurately weigh about 500 mg, dissolve in 50 ml of *glacial acetic acid*, mix, add *crystal violet*, and titrate with *0.1 M perchloric* acid. Each ml of *0.1 M perchloric acid* is equivalent to 12.92 mg of $C_8H_{19}N$. Not less than 98 per cent is found.

Dimethicone: Of the Indian Pharmacopoeia.

2,5-Dimethoxybenzaldehyde: $C_9H_{10}O_3$=166.18

General laboratory reagent grade of commerce.

mp, about 50°.

Dimethylacetamide; *N, N*-dimethylacetamide: C_4H_9NO = 87.12

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 165°; wt. per ml, about 0.94 g.

Dimethylamine: *N*-Methylmethanamine: C_2H_7N = 45.08

Use analytical grade of commerce.

Dimethylamine solution: A 40 per cent w/w solution of *dimethylamine* in *water*.

4-Dimethylaminobenzaldehyde; *p*-Dimethylamino-benzaldehyde; Dimethylaminobenzaldehyde: $C_9H_{11}NO$ = 149.19

Analytical reagent grade of commerce.

White or pale yellow, crystalline powder; mp, about 74°.

Dimethylaminobenzaldehyde solution: Dissolve 0.2 g of *4-dimethylaminobenzaldehyde* in 20 ml of *ethanol* (95 per cent) and add 0.5 ml of *hydrochloric acid*. Shake the solution with *activated charcoal* and filter. The colour of the solution is not more intense than that of freshly prepared *0.0001 M iodine*. Prepare immediately before use.

Dimethylaminobenzaldehyde Reagent: Dissolve 0.125 g of *4-dimethylaminobenzaldehyde* in a cooled mixture of 65 ml of *sulphuric acid* and 35 ml of *water* and add 0.1 ml of *ferric chloride test solution*. Allow to stand for 24 hours in the dark before use.

When stored at room temperature, it must be used within one week. It may be stored in a refrigerator for several months. Discard the solution when a yellow colour develops.

Dimethylaminobenzaldehyde Solution, Ethanolic; Alcoholic Dimethylaminobenzaldehyde Solution: Dissolve 1 g of *4-dimethylaminobenzaldehyde* in 30 ml of *ethanol (95 per cent)* and add 180 ml of *1-butanol* and 30 ml of *hydrochloric acid*.

4-Dimethylaminocinnamaldehyde: $C_{11}H_{13}NO = 175.23$

General laboratory reagent grade of commerce.

Orange or orange brown crystals or powder; mp, about 138°.

Dimethylaniline; *N,N*-Dimethylaniline: $C_8H_{11}N = 121.18$

Analytical reagent grade of commerce.

Clear liquid which darkens on storage; bp, about 193°; refractive index at 20°, 1.557 to 1.5591; wt. per ml, about 0.96 g.

2,3 Dimethylaniline; 2,3-Xylidine: $C_8H_{11}N = 121.18$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 221°; refractive index at 20°, about 1.568; wt. per ml, about 0.99 g.

2,6-Dimethylaniline; 2,6-Xylidine: $C_8H_{11}N = 121.18$

General laboratory reagent grade of commerce.

Colourless liquid; wt. per ml, about 0.98.

1,1-Dimethylethyl Methyl Ether; *tert*-Butyl methyl ether: $C_5H_{12}O = 88.1$

General reagent grade of commerce.

Minimum transmittance of 50 per cent at 240 nm, 80 per cent at 255 nm and 98 per cent at 280 nm using *water* in the reference cell; refractive index at 20°, about 1.376.

Dimethylformamide: $C_3H_7NO = 73.09$

Analytical reagent grade of commerce.

Colourless liquid; bp, about 153°; wt per ml, about 0.95 g.

Complies with the following additional test.

WATER (2.3.43) — Not more than 0.1 per cent.

Dimethylformamide Solution (5 per cent v/v): Dilute 5 ml of *dimethylformamide* to 100 ml with *water*.

Dimethyl Glutaric Acid: $C_7H_{12}O_4 = 160.17$

General laboratory reagent grade of commerce.

Dimethylglyoxime; Diacetyldioxime; 2,3-Butanedione dioxime: $C_4H_8N_2O_2 = 116.12$

Analytical reagent grade of commerce.

White, crystalline powder or colourless crystals; mp, about 240° with decomposition; sulphated ash, not more than 0.05 per cent.

***N,N*-dimethyloctylamine**: $C_{10}H_{23}N = 157.30$

General laboratory reagent grade of commerce.

3, 4-Dimethylphenol: $C_8H_{10}O = 122.2$

General laboratory reagent grade of commerce.

mp, about 27°, bp, about 65°.

***N,N*-Dimethyl-*p*-phenylenediamine Sulphate**;

N,N-Dimethyl-4-phenylenediamine sulphate: $C_8H_{12}N_2H_2SO_4 = 234.27$

General laboratory reagent grade of commerce.

***N,N*-Dimethyl-*p*-phenylenediamine Sulphate Solution**:

Boil 25 g of *N,N-dimethyl-p-phenylenediamine sulphate* with 600 ml of *ethanol (90 per cent)* under a reflux condenser. When dissolved add *activated charcoal*, mix well and filter whilst hot. Allow to stand overnight and then cool in ice, filter through sintered glass. Wash with ice-cold *ethanol* until free from colour and dry at room temperature at a pressure of 2 kPa. Dissolve 50 mg of the recrystallised material in sufficient *water* to produce 50 ml.

Dimethyl Phthalate: $C_{10}H_{10}O_4 = 194.19$

General laboratory reagent grade of commerce.

Colourless or faintly coloured liquid; wt. per ml, about 1.19 g; contains not less than 99.0 per cent and not more than 100.5 per cent w/w of $C_{10}H_{10}O_4$.

ASSAY — Carry out the method for the determination of esters (2.3.25), using 1.5 g and 50 ml of *0.5 M ethanolic potassium hydroxide*. 1 ml of *0.5 M ethanolic potassium hydroxide* is equivalent to 0.04855 g of $C_{10}H_{10}O_4$.

***N,N'*-Dimethylpiperazine**; Dimethylpiperazine; 1,4-Dimethylpiperazine: $C_6H_{14}N_2 = 114.20$

General laboratory reagent grade of commerce.

Colourless liquid with a characteristic odour; bp, about 131°; wt per ml, about 0.84 g.

Dimethyl Sulphoxide: $(CH_3)_2SO = 78.13$

General Laboratory reagent grade of commerce.

Clear, colourless, viscous liquid, odourless or with a slight, but unpleasant odour; hygroscopic; bp about 192°; wt per ml, about 1.103 g.

1,3-Dinitrobenzene: $C_6H_4(NO_2)_2 = 168.11$

General laboratory reagent grade of commerce.

Pale yellow needles; mp, about 90°.

Dinitrobenzene Solution: A 1 per cent w/v solution of *1,3-dinitrobenzene* in *ethanol (95 per cent)*.

3,5-Dinitrobenzoic Acid; Dinitrobenzoic Acid: $C_7H_4N_2O_6 = 212.12$

General laboratory reagent grade of commerce.

Almost colourless crystals; mp, about 206°.

Dinitrobenzoic Acid Solution: A 2.0 per cent w/v solution of *3,5-dinitrobenzoic acid* in *ethanol (95 per cent)*.

2,4-Dinitrofluorobenzene Reagent. 10 mg per ml of *2,4-dinitrofluorobenzene* in *alcohol*. This solution may be used for 5 days keep in refrigerator when not in use.

2,4-Dinitrophenylhydrazine; Dinitrophenylhydrazine: $C_6H_6N_4O_4 = 198.14$

Analytical reagent grade of commerce.

Reddish orange crystals or crystalline powder; mp, about 203°.

Dinitrophenylhydrazine-Aceto-Hydrochloric Acid Solution; Dinitrophenylhydrazine Reagent: Dissolve 0.2 g of *2,4 dinitrophenylhydrazine* in 20 ml of *methanol* and add 80 ml of a mixture of equal volumes of *7 M hydrochloric acid* and *5 M acetic acid*.

Prepare immediately before use.

Dinitrophenylhydrazine Solution: Dissolve 1.5 g of *2, 4-dinitrophenylhydrazine* in 20 ml of *sulphuric acid (50 per cent v/v)*. Dilute to 100 ml with *water* and filter. The solution must be freshly prepared.

Dioctyl Sodium Sulphosuccinate: $C_{20}H_{37}NaO_7S = 444.56$

General laboratory reagent grade of commerce.

White, waxy flakes; usually contains about 90 per cent of $C_{20}H_{37}NaO_7S$.

Dioxan; 1,4-Dioxan; Diethylene Dioxide: $C_4H_8O_2 = 88.11$

Analytical reagent grade of commerce.

Colourless liquid with an ethereal odour; bp, about 101°; wt. per ml, about 1.03 g.

Do not distil unless the dioxan complies with the test for peroxides.

PEROXIDES — Place 8 ml of *starch iodide solution* in a 12 ml glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the reagent under examination, shake vigourously and allow to stand protected from light for 30 minutes; no colour is produced.

Diphenyl: $C_{12}H_{10} = 154.21$

General laboratory reagent grade of commerce.

White crystals or crystalline powder; odour, characteristic; mp, about 70°.

Diphenylamine: $(C_6H_5)_2NH = 169.23$

Analytical reagent grade of commerce.

White crystals; odour, characteristic; mp, about 55°.

Store protected from light.

Diphenylamine Solution: A 0.1 per cent w/v solution of *Diphenylamine* in *sulphuric acid*.

Store protected from light.

9,10-diphenylanthracene; Diphenylanthracene: $C_{26}H_{18} = 330.43$.

General laboratory reagent grade of commerce.

mp, about 248°.

***N,N'*-Diphenylbenzidine**: Diphenylbenzidine: $C_{24}H_{20}N_2 = 336.42$

General laboratory reagent grade of commerce.

White or faintly grey, crystalline powder; mp, about 248°.

Complies with the following test.

NITRATE — Dissolve 8 mg in a cooled mixture of 45 ml of *nitrogen free sulphuric acid* and 5 ml of *water*; the solution is colourless or very pale blue.

Store protected from light.

Diphenylboric Acid Aminoethyl Ester:

White or slightly yellow, crystalline powder,

Practically insoluble in *water*, soluble in 95 per cent *ethanol*.

1,5-Diphenylcarbazide: Diphenylcarbazide: $C_{13}H_{14}N_4O = 242.28$

Analytical reagent grade of commerce.

White, crystalline powder gradually turning pink on exposure to air; mp, about 170°.

Store protected from light.

Diphenylcarbazide Solution: Dissolve 0.2 g of *1,5-diphenylcarbazide* in 10 ml of *glacial acetic acid* and dilute to 100 ml with *ethanol*.

Prepare immediately before use.

1,5-Diphenylcarbazone; Diphenylcarbazone; $C_{13}H_{12}N_4O = 240.26$

General laboratory reagent grade of commerce.

Orange, crystalline powder; mp, about 157°, decomposition.

Diphenylcarbazone Mercuric Reagent; Diphenylcarbazone-Mercury Reagent: Dissolve 0.1 g of *1,5 diphenylcarbazone* in sufficient *ethanol* to produce 50 ml and, separately, dissolve 1 g of *mercuric chloride* in sufficient *ethanol* to produce 50 ml. Mix equal volumes of the two solutions.

Dipotassium Hydrogen Phosphate: Dipotassium Hydrogen Orthophosphate; Dibasic Potassium Phosphate: $K_2HPO_4 = 174.17$

Analytical reagent grade of commerce.

White granules; somewhat hygroscopic.

Dipotassium Hydrogen Phosphate, 0.1 M: Dissolve 17.417 g of *dipotassium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Dipotassium Tartrate; Potassium tartrate: $C_4H_4K_2O_6,½H_2O$ = 235.3

White or almost white, granular powder or crystals.

Disodium Arsenate; Disodium hydrogen arsenate heptahydrate; dibasic sodium arsenate; sodium arsenate heptahydrate: $Na_2HAsO_4,7H_2O$ = 312.0

Crystals, efflorescent in warm air, freely soluble in water, soluble in glycerol, slightly soluble in *ethanol (95 per cent)*. The aqueous solution is alkaline to litmus.

d_{20}^{20}, about 1.87.

mp, about 57°, when rapidly heated.

Disodium Chromotropate;(4,5-Dihydroxy-2,7-naphthalenedi-sulphonic Acid, Disodium Salt);chromo tropic acid disodium salt: $C_{10}H_6O_8S_2Na_2,2H_2O$ = 400.29

Analytical reagent grade of commerce.

Disodium Edetate: Of the Indian Pharmacopoeia.

Disodium Edetate, x M: Solutions of any molarity x M may be prepared by dissolving 372.2 x g of *disodium edetate* in sufficient *water* to produce 1000 ml.

Disodium Hydrogen Phosphate; Disodium Hydrogen Orthophosphate: See Sodium Phosphate of the Indian Pharmacopoeia.

Disodium Hydrogen Phosphate x M: Solutions of any molarity xM may be prepared by dissolving 141.95 x g of *disodium hydrogen phosphate* in sufficient *water* to produce 1000 ml.

Disodium Hydrogen Phosphate, Anhydrous; Disodium Hydrogen Orthophosphate, Anhydrous; Anhydrous Sodium Phosphate: Na_2HPO_4 = 141.95

Analytical reagent grade of commerce.

Hygroscopic powder; absorbs 2 to 7 mols of H_2O when exposed to air.

Store protected from moisture.

Disodium Hydrogen Phosphate, Dihydrate; Disodium Hydrogen Orthophosphate Dihydrate; Sodium Phosphate, Dibasic, Dihydrate; Disodium hydrogen phosphate, dihydrate : $Na_2HPO_4,2H_2O$ = 178.0

Analytical reagent grade of commerce.

Disodium Hydrogen Phosphate Solution: A 10.0 per cent w/v *solution of disodium hydrogen phosphate* in *water*.

Disodium 1-nitroso-2-naphthol-3,6-disulphonate: $C_{10}H_5NNa_2O_8S_2$ = 377.3

Yellow crystals and crystalline powder.

5,5'-Dithiobis(2-nitrobenzoic acid); 3-Carboxy-4-nitrophenyl Disulphide; Ellman's Reagent: $C_{14}H_8N_2O_8S_2$ = 396.35

Yellow powder; mp, about 242°

Dithiothreitol: $C_4H_{10}O_2S_2$ = 154.2

Slightly hygroscopic needles from either, mp 42° to 43°.

Dithizone; 1,5-Diphenylthiocarbazone; Diphenylthiocarbazone: C_6H_5N:NCSNHNHC$_6H_5$ = 256.32

Analytical reagent grade of commerce.

Almost black powder; mp, about 168°, with decomposition.

Store protected from light.

Dithizone-Carbon Tetrachloride Solution: Dissolve 10 mg of *dithizone* in 1000 ml of *carbon tetrachloride*. Prepare this solution fresh for each determination.

Dithizone Extraction Solution: Dissolve 30 mg of *dithizone* in 1000 ml of *chloroform* and add 5 ml of *ethanol (95 per cent)*. Store the solution in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of a 1 per cent v/v solution of *nitric acid* and discard the acid.

Dithizone solution: A 0.05 per cent w/v solution of *dithizone* in *chloroform*.

Prepare immediately before use.

Dithizone Standard Solution: Dissolve 10 mg of *dithizone* in 1000 ml of *chloroform*. Store the solution in a glass-stoppered, lead-free, light-resistant bottle in a refrigerator.

Dodecyltrimethylammonium Bromide: $C_{15}H_{34}NBr$ = 308.3

General reagent grade of commerce; mp, about 246° with decomposition.

Domiphen Bromide; Dodecyldimethyl-2-phenoxy-ethyl-ammonium bromide: $C_{22}H_{40}BrNO$ = 414.47

Colourless or faintly yellow, crystalline flakes; mp, about 111°; contains not less 97.0 per cent and not more than 100.5 per cent of $C_{22}H_{40}BrNO$, calculated with reference to the dried substance.

ASSAY — Dissolve 2 g of *domiphen bromide* in sufficient *water* to produce 100 ml. Transfer 25 ml to a separating funnel and add 25 ml of *chloroform*, 10 ml of *0.1 M Sodium hydroxide* and 10 ml of a freshly prepared 5 per cent w/v solution of *potassium iodide*. Shake well, allow to separate and discard the chloroform layer. Wash the aqueous layer with three quantities, each of 10 ml, of *chloroform* and discard the chloroform solutions. Add 40 ml of *hydrochloric acid*, allow to cool and titrate with *0.05 M potassium iodate* until the deep brown colour is discharged. Add 2 ml of *chloroform* and continue the titration shaking vigorously until the chloroform layer no longer changes colour. Perform a blank determination using a mixture of 10 ml of the freshly prepared *potassium iodide solution*, 20 ml of *water* and 40 ml of *hydrochloric acid*. The difference between the titrations represents the amount of *potassium iodate* required.

1 ml of *0.05 M Potassium iodate* is equivalent to 0.04145 g of $C_{22}H_{40}BrNO$.

Dotriacontane: $C_{32}H_{66}$ = 450.87

General laboratory reagent grade of commerce.

White plates; mp, about 69°.

IMPURITIES — Not more than 0.1 per cent of impurities with the same retention time as α-tocopherol acetate when determined by gas chromatography (2.4.13).

Dragendorff Reagent

SOLUTION I — Dissolve 0.85 g of *bismuth subnitrate* in 10 ml of *glacial acetic acid* and 40 ml of *water* with the aid of heat and filter, if necessary.

SOLUTION II — Dissolve 8 g of *potassium iodide* in 30 ml of *water*.

Stock solution: Solutions I and II are equally mixed.

Spray reagent: 1 ml of stock solution is mixed with 2 ml of *glacial acetic acid* and 10 ml of *water*.

Prepare immediately before use.

Electrolyte reagent for the determination of water: Commercially available anhydrous reagent or a combination of anhydrous reagents for the coulometric titration of *water*, containing suitable organic basis, *sulphur dioxide* and *iodide* dissolved in a suitable solvent.

Endoproteinase Lys-C Solution: Microbial extracellular proteolytic enzyme secreted by Achromobacterlyticus. A lyophilised powder, free of salts.

Ergocalciferol: Of the Indian Pharmacopoeia.

Ethanesulfonic acid: $C_2H_6O_3S$ = 110.13

bp, 122 to 123° at 0.01 mm Hg

Ethanol; Alcohol, Dehydrated: Of the Indian Pharmacopoeia.

Ethanol (95 per cent): of the Indian Pharmacopoeia.

Ethanol (x per cent): Diluted *ethanol*s may be prepared by diluting the volumes of *ethanol (95 per cent)* indicated in the following table to 1000 ml with *water*.

Strength per cent v/v	Volume of *ethanol (95 per cent)* (ml)	Weight per ml (g)
90	947	0.83
80	842	0.86
75	789	0.88
70	737	0.89
60	632	0.91
50	526	0.93
40	421	0.95
25	263	0.97
20	210	0.976

Ethanol, Aldehyde-free; Aldehyde-free Ethanol (95 per cent): Mix 1200 ml of *ethanol (95 per cent)* with 5 ml of a 40 per cent w/v solution of *silver nitrate* and 10 ml of a cooled 50 per cent w/v solution of *sodium hydroxide*. Shake, allow to stand for a few days and filter. Distill the filtrate immediately before use.

Complies with the following additional test.

ALDEHYDE — To 25 ml contained in a 300 ml flask add 75 ml of *dinitrophenylhydrazine solution*, heat on a water-bath under a reflux condenser for 24 hours, remove the *ethanol* by distillation, dilute to 200 ml with a 2 per cent v/v solution of *sulphuric acid* and set aside for 24 hours; no crystals are produced.

Ethanolamine: Of the Indian Pharmacopoeia.

Ether; Diethyl Ether; Solvent Ether, Ethyl Ether: $(C_2H_5)_2O$ = 74.12

Analytical reagent grade of commerce.

Volatile, highly flammable colourless liquid; bp, about 34°; wt. per ml, about 0.71 g.

NOTE — Do not determine the boiling range or distil unless the ether complies with the test for peroxides.

PEROXIDES — Place 8 ml of *starch iodide solution* in a 12 ml glass stoppered cylinder about 1.5 cm in diameter. Fill completely with the reagent under examination shake vigorously and allow to stand protected from light for 30 minutes; no colour is produced.

Store protected from light at a temperature not exceeding 15°. The name and concentration of any added stabilizer are stated on the label.

Ether, Peroxide-free: Shake 1000 ml of *ether* with 20 ml of a solution of 30 g of *ferrous sulphate* in 55 ml of *water* and 3 ml of *sulphuric acid*. Continue shaking until a small sample no longer produces a blue colour when shaken with an equal volume of a 2 per cent w/v solution of potassium *iodide* and 0.1 ml of *starch mucilage*.

***p*-Ethoxychrysoidine Hydrochloride**: 4-p-Ethoxyphenylazo-m-phenylenediamine Hydrochloride: $C_{14}H_{16}N_4O$, HCl = 292.76

General laboratory reagent grade of commerce.

Red powder.

Ethoxychrysoidine Hydrochloride Solution: A 0.1 per cent w/v solution of *p-ethoxychrysoidine hydrochloride* in *ethanol (95 per cent)*.

Complies with the following test.

SENSITIVITY TO BROMINE — To a mixture of 0.05 ml and 5 ml of *2 M hydrochloric acid* add 0.05 ml of *0.0167 M bromine*; the colour changes from red to light yellow within 2 minutes.

Ethyl Acetate: $CH_3COOC_2H_5$ = 88.11

Analytical reagent grade of commerce.

Colourless liquid with a fruity odour; bp, about 77°; wt. per ml, about 0.90 g.

Ethyl Benzoate: $C_9H_{10}O_2 = 150.2$

General laboratory reagent grade of commerce.

A colourless liquid; n_D^{20}, about 1.505; wt. per ml, about 1.05 g.

Ethyl Cyanoacetate: $CNCH_2CO_2C_2H_5 = 113.12$

Analytical reagent grade of commerce.

Colourless or almost colourless liquid; wt. per ml, about 1.06 g.

Ethylenediamine; 1,2-Diaminoethane: $NH_2CH_2CH_2NH_2 = 60.10$

General laboratory reagent grade of commerce.

Colourless, clear, thick liquid with ammonical odour; bp, about 118°; refractive index at 20°, about 1.457; wt. per ml, about 0.9 g.

Ethylene Glycol; 1,2-Ethanediol; Ethane-1,2-diol: $C_2H_6O_2$= 62.07

General laboratory reagent grade of commerce.

Slightly viscous liquid; taste, sweet; considerably hygroscopic; bp, about 198°.

Ethylene Oxide; Oxirane: $C_2H_4O = 44.0$

General laboratory reagent grade of commerce.

A colourless gas, liquefaction point, about 12°.

***N*-Ethylglucamine**; Eglumine; 1-Deoxy-1-ethylamino-D-glucitol: $C_8H_{19}O_5 = 209.24$.

General commercial grade.

mp, about 138°.

***N*-Ethylglucamine Hydrochloride**: $C_8H_{19}O_5,HCl = 245.70$.

General commercial grade.

mp, about 132°.

2-Ethylhexanoic Acid; 2- Ethylhexoic Acid: $C_8H_{16}O_2 = 144.21$

General laboratory reagent grade of commerce.

Colourless liquid; wt. per ml, about 0.91 g; refractive index at 20°, about 1.425.

Complies with the following test.

RELATED SUBSTANCES — Determine by gas chromatography (2.4.13).

Using 1 µl of a solution prepared in the following manner. Suspend 0.2 g in 5 ml of *water*, add 3 ml of *2 M hydrochloric acid* and 5 ml of *hexane*, shake for 1 minute, allow the layers to separate and use the upper layer.

The chromatographic system described in the test for 2-Ethylhexanoic Acid in the monograph for Amoxycillin Sodium may be used.

The sum of the areas of any secondary peaks is not greater than 2.5 per cent of the area of the principal peak.

***N*-ethylmaleimide**: $C_6H_7NO_2 = 125.12$

General laboratory reagent grade of commerce. mp, about 43° to 46°.

2-Ethyl-2-methylsuccinic Acid; 2-Ethyl-2-methyl-butanedioic Acid: $C_7H_{12}O_4$=160.17

General reagent grade of commerce.

mp, about 106°.

Ethyl Paraben: Of the Indian Pharmacopoeia.

Eugenol; 4-Allyl-2-methoxyphenol: $C_{10}H_{12}O_2 = 164.20$

General laboratory reagent grade of commerce.

Colourless or pale yellow, oily liquid; wt. per ml, about 1.07 g.

For the determination of eugenol in volatile oils, use a grade of commerce containing not less than 99.0 per cent of $C_{10}H_{12}O_2$.

Store protected from light.

Euglobulins, Bovine: For the preparation, use fresh bovine blood collected into an anticoagulant solution (for example sodium citrate solution). Discard any haemolysed blood. Centrifuge at 1500 to 1800 *g* between 15° and 20° to obtain a supernatant plasma poor in platelets.

To 1 litre of the bovine plasma add 75 g of *barium sulphate* and shake for 30 minutes. Centrifuge at 1500 to 1800 g between 15° and 20° and draw off the clear supernatant liquid. Add 10 ml of a 0.02 per cent w/v solution of *aprotinin* and shake to ensure mixing. In a container with a minimum capacity of 30 litres in a chamber at 4° introduce 25 litres of *distilled water* at 4° and add about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant liquid obtained from the plasma; a white precipitate is produced. Allow to settle at 4° for 10 to 15 hours. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifugation at 4°. Suspend the precipitate by dispersing mechanically in 500 ml of *distilled water* at 4°, shake for 5 minutes and collect the precipitate by centrifugation at 4°. Disperse the precipitate mechanically in 60 ml of a solution containing 0.9 per cent w/v of *sodium chloride* and 0.09 per cent w/v of *sodium citrate* and adjust the pH to 7.2 to 7.4 by adding a 1 per cent w/v solution of *sodium hydroxide*. Filter through a sintered-glass filter; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable implement. Wash the filter and the implement with 40 ml of the chloride-citrate solution described above and dilute to 100 ml with the same solution. Freeze-dry the solution. The yields are generally 6 to 8 g of euglobulins per litre of bovine plasma.

Complies with the following test.

SUITABILITY TEST — For this test, prepare the solutions using *phosphate buffer pH 7.4* containing 3 per cent w/v of *bovine*

albumin. In to a test-tube, 8 mm in diameter placed in a water-bath at 37°, introduce 0.2 ml of a solution of a reference preparation of urokinase containing 100 Units of urokinase activity per ml and 0.1 ml of a solution of *thrombin* containing 20 Units per ml. Add rapidly 0.5 ml of a solution containing 10 mg of the euglobulin fraction per ml; a firm clot is produced in less than 10 seconds. Note the time that elapses between the addition of the solution of the euglobulin fraction and the lysis of the clot. The lysis time does not exceed 15 minutes.

Store protected from moisture at 4°and use within 1 year.

Euglobulins, Human: For the preparation, use fresh human blood collected into an anticoagulant solution (for example sodium citrate solution) or human blood for transfusion collected into plastic blood bags that has just reached its expiry date. Discard any haemolysed blood. Centrifuge at 1500 to 1800 g at 15° to obtain a supernatant plasma poor in platelets. Iso-group plasmas may be mixed.

To 1 litre of the human plasma add 75 g of *barium sulphate* and shake for 30 minutes. Centrifuge at not less than 15000 g at 15° and draw off the clear supernatant liquid. Add 10 ml of a 0.02 per cent w/v solution of *aprotinin* and shake to ensure mixing. In a container with a minimum capacity of 30 litres in a chamber at 4° introduce 25 litres of *distilled water* at 4° and add about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant liquid obtained from the plasma; a white precipitate is produced. Allow to settle at 4° for 10 to 15 hours. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4°. Suspend the precipitate by dispersing mechanically in 500 ml of distilled *water* at 4°, shake for 5 minutes and collect the precipitate by centrifuging at 4°. Disperse the precipitate mechanically in 60 ml of a solution containing 0.9 per cent w/v of *sodium chloride* and 0.09 per cent w/v of *sodium citrate* and adjust the pH to 7.2 to 7.4 by adding a 1 per cent w/v solution of *sodium hydroxide*. Filter through a sintered-glass filter; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable implement. Wash the filter and the implement with 40 ml of the chloride-citrate solution described above and dilute to 100 ml with the same solution. Freeze dry the solution. The yields are generally 6 to 8 g of euglobulins per litre of human plasma.

Complies with the following test.

SUITABILITY TEST — Carry out the test described under Bovine Euglobulins using *citro-phosphate buffer pH 7.2* containing 3 per cent w/v of *bovine albumin*.

Store in tightly-closed containers at 4° and use within 1 year.

Ferric Ammonium Sulphate; Ammonium Iron (III) Sulphate; Ferric Alum: $Fe(NH_4)(SO_4)_2,12H_2O = 482.18$

Analytical reagent grade of commerce.

Pale violet crystals or nearly colourless crystalline powder.

Ferric Ammonium Sulphate Solution; Ammonium Iron (III) Sulphate Solution: An 8.0 per cent w/v solution of *ferric ammonium sulphate*.

Ferric Ammonium Sulphate Solution, Acid: Dissolve 0.2 g of *ferric ammonium sulphate* in 50 ml of *water*, add 6 ml of *dilute nitric acid* and add sufficient *water* to produce 100 ml.

Ferric Chloride; Anhydrous Ferric Chloride; Iron(III) Chloride; Anhydrous Iron(III) Chloride: $FeCl_3 = 162.22$

General laboratory reagent grade of commerce.

Greenish black crystals or crystalline powder, free from the orange colour of the hydrated salt, which is readily acquired by exposure to atmospheric moisture.

Ferric Chloride-Ferricyanide-Arsenite Solution

SOLUTION I — Dissolve 2.7 g of *ferric chloride hexahydrate* in 100 ml of *2 M of hydrochloric acid*.

SOLUTION II — Dissolve 3.5 g of *potassium ferricyanide* in 100 ml of *water*.

SOLUTION III — Dissolve 3.8 g of *arsenic trioxide* in 25 ml of hot *2 M sodium hydroxide*. Allow to cool, add 50 ml of *1 M sulphuric acid* and dilute with *water* to 100 ml.

Immediately before use mix 5 volumes of solution I, 5 volumes of solution II and 1 volume of solution III.

Ferric Chloride Hexahydrate; Iron(III) Chloride Hexahydrate: $FeCl_3,6H_2O = 270.32$

Analytical reagent grade of commerce.

Brownish yellow or orange monoclinic crystals; very hygroscopic.

Ferric Chloride Solution: A solution prepared from *ferric chloride hexahydrate* so as to contain about 15 per cent w/v of $FeCl_3$.

Ferric Chloride Test Solution: A 5.0 per cent w/v solution of *ferric chloride*.

Ferric Nitrate; Iron(III) Nitrate: $Fe(NO_3)_3,9H_2O = 404.0$

General Laboratory reagent grade of commerce.

Light-purple crystals or crystalline mass.

Ferric Nitrate Solution; Iron(III) Nitrate Solution: A 0.1 per cent w/v solution of *ferric nitrate* in 0.1 per cent v/v solution of *nitric acid*.

Ferric Sulphate Pentahydrate; Iron(III) Sulphate Pentahydrate: $Fe_2(SO_4)_3,5H_2O = 489.9$

Analytical reagent grade of commerce.

White to yellowish powder.

Store in well-closed, light-resistant containers.

Ferrous Ammonium Sulphate; Ammonium Iron(II) Sulphate: $Fe(NH_4)_2(SO_4)_2,6H_2O = 392.13$

Analytical reagent grade of commerce.

Pale greenish blue crystals or crystalline powder.

Store in well-closed, light-resistant containers.

Ferrous Sulphate: Of the Indian Pharmacopoeia.

Ferrous Sulphate-Citrate Solution: Dissolve 1 g of *sodium metabisulphite* in 200 ml of *water*, add 1 ml of *1 M hydrochloric acid*, 1.5 g of *ferrous sulphate* and 10 g of *sodium citrate*.

The solution must be freshly prepared.

Ferrous Sulphate Solution: A 2.0 per cent w/v solution of *ferrous sulphate* in freshly boiled and cooled *water*.

The solution must be freshly prepared.

Fibrin: The insoluble protein which separates during the coagulation of blood, washed and shredded.

Fluorescamine: $C_{17}H_{10}O_4 = 278.3$

General laboratory reagent grade of commerce.

mp, about, 155°.

Fluorescein; CI 45350; 3',6'-Fluorandiol; 3',6'-Dihydroxyfluoran; Solvent Yellow 94; 3',6'-Dihydroxyspiro [isobenzofuran-1(3*H*),9'-[9*H*]xanthen] 3-one; 9'-(*o*-carboxyphenyl)-6-hydoxy-3*H*-xanthen-3-one: $C_{20}H_{12}O_5$ = 332.30

General laboratory reagent grade of commerce.

Yellowish red to red powder; mp, about 315°, with decomposition.

Fluorescein Sodium: Of the Indian pharmacopoeia.

1-Fluoro-2, 4-dinitrobenzene;
2,4-Dinitrofluorobenzene: $C_6H_3FN_2O_4 = 186.10$

General laboratory reagent grade of commerce.

Pale yellow, vesicatory crystals, lumps or liquids with a lachrymatory vapour; refractive index at 20°, about 1.569; wt. per ml, about 1.48 g.

1-Fluoro-2, 4-dinitrophenyl-5-*L*-alaninamide; N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide; Marfey's reagent; FDAA: $C_9H_9FN_4O_5 = 272.2$

Yellow or orange powder; mp: about 228°.

Enantiomeric purity: Minimum 99.5 per cent.

Fluoxymesterone; 9'-Fluoro-11b,17b-dihydroxy-17a-methylandrost-4-en-3-one: $C_{20}H_{29}FO_3 = 336.45$

General laboratory reagent grade of commerce.

mp, about 229°.

Folic Acid: Of the Indian Pharmacopoeia.

Formaldehyde Solution; Formalin: $HCHO = 30.03$

Analytical reagent grade of commerce.

Colourless, aqueous solution with a lachrymatory vapour; wt. per ml, about 1.08 g; contains not less than 34.0 per cent w/v and not more than 37.0 per cent w/v of CH_2O.

ASSAY — Dilute 5 ml to 1000 ml with *water*. To 10 ml of the solution add 25.0 ml of *0.05 M iodine* and 10 ml of *1 M sodium hydroxide*. Allow to stand for 5 minutes, add 12 ml of *1 M hydrochloric acid* and titrate the excess of *iodine* with *0.1 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator. Perform a blank determination and make any necessary correction.

1 ml of *0.05 M iodine* is equivalent to 0.0015 g of CH_2O.

Store protected from moisture, preferably at a temperature not below 15°.

Formaldehyde Solution, Dilute: Dilute 34 ml of *formaldehyde solution* with sufficient *water* to produce 100 ml.

Formamide: $HCONH_2 = 45.04$

Analytical reagent grade of commerce.

Colourless, oily liquid; wt. per ml, about 1.13 g.

Formic Acid: $HCOOH = 46.03$

Analytical reagent grade of commerce.

Colourless liquid; odour, very pungent; highly corrosive; wt. per ml, about 1.20 g; contains about 90.0 per cent w/w of CH_2O_2 and is about 23.6 M in strength.

Formic Acid, 15 M: Dilute 63.3 ml of *formic acid* to 100 ml with *water*.

Formic Acid, Anhydrous: $HCOOH = 46.03$

Analytical reagent grade of commerce.

Colourless liquid; odour, pungent; highly corrosive; wt. per ml, about 1.22 g; contains not less than 98.0 per cent w/w of HCOOH.

ASSAY — Weigh accurately a conical flask containing 10 ml of *water*, quickly add about 1 ml of the reagent under examination and weigh again. Add 50 ml of *water* and titrate with *1 M sodium hydroxide* using 0.5 ml of *phenolphthalein solution* as indicator. 1 ml of *1 M sodium hydroxide* is equivalent to 0.04603 g of HCOOH.

D-Fructose; Laevulose: $C_6H_{12}O_6 = 180.16$

General laboratory reagent grade of commerce.

White, crystalline powder; mp, about 103°, with decomposition; $[\alpha]_D^{20}$, about 92° (10 per cent w/v in *water* containing 0.05 ml of *5 M ammonia*).

Furfuraldehyde; Furfural; furan-2-aldehyde: $C_5H_4O_2 = 96.09$

General laboratory reagent grade of commerce.

A colourless or pale brownish yellow, oily liquid; bp, about 162°; 1.155 to 1.161.

Fumaric Acid: Of the Indian Pharmacopoeia.

Fusidic Acid: Of the Indian Pharmacopoeia.

Gastric Juice, Artificial: Dissolve 2.0 g of *sodium chloride* and 3.2 g of *pepsin* in *water*. Add 80 ml of *1 M hydrochloric acid* and dilute to 1000 ml with *water*.

D-Galactose: $C_6H_{12}O_6$

General laboratory reagent grade of commerce.

White, crystalline powder; mp, about 164°; $[\alpha]_D^{20}$, about + 80° (10 per cent w/v in *water* containing 0.05 ml of *5 M ammonia*).

Gelatin: Of the Indian Pharmacopoeia.

Gelatin Capsule Shells, Hard: Of the Indian Pharmacopoeia.

Gelatin, Hydrolysed: Dissolve 50 g of gelatin in 1000 ml of *water*, heat in saturated steam at 121° for 90 minutes and freeze-dry.

Gelatin, Pancreatic Digest of: Microbiological reagent grade of commerce.

Gitoxin: $C_{41}H_{64}O_{14} = 780.92$

General laboratory reagent grade of commerce.

White, crystalline powder; mp, about 283°, with decomposition; $[\alpha]_D^{20}$, about +22° (0.5 per cent w/v in a mixture of equal volumes of *chloroform* and *methanol*).

Complies with the following additional test.

HOMOGENEITY — Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 7.5 volumes of *water*, 10 volumes of *methanol* and 75 volumes of *ethyl acetate*.

Test solution. Add 20 ml of *ethanol (50 per cent)* and 10 ml of *lead acetate solution* to 1.0 g, in *No.180 powder*, boil for 2 minutes and allow to cool. Centrifuge and extract the clear supernatant liquid with two quantities, each of 15 ml of *chloroform*, separating the layers, if necessary, by centrifugation. Dry the combined extracts over *anhydrous sodium sulphate* and filter. Evaporate 10 ml of this solution to dryness on a water-bath and dissolve the residue in 1 ml of a mixture of equal volumes of *chloroform* and *methanol*.

Apply to the plate 20 µl of the solution as a band, 2 cm by 3 mm. Allow the mobile phase to rise 10 cm. Dry the plate, allow the solvents to evaporate, spray with a mixture of 8 volumes of a 25 per cent w/v solution of *trichloroacetic acid* in *ethanol (95 per cent)* and 2 volumes of a 1 per cent w/v solution of *chloramine T*, heat at 105° for 10 minutes and examine under ultra-violet light at 365 nm. The chromatogram shows only one light blue fluorescent band due to gitoxin.

Glutamic acid: $C_5H_9NO_4 = 147.1$

A white, crystalline powder or colourless crystals.

Glutaric Acid; Pentanedioic acid: $C_5H_8O_4 = 132.1$

White, crystalline powder.

Glycine: Of the Indian Pharmacopoeia.

Glycerin: Of the Indian Pharmacopoeia.

Glycerin (85 per cent): Glycerin containing 12.0 to 16.0 per cent w/w of *water*; wt. per ml, 1.22 to 1.24 g.

Glycollic Acid; 2-Hydroxyacetic Acid: $C_2H_4O_3 = 76.05$

General laboratory reagent grade of commerce.

mp, about 80°.

Glycyrrhetinic Acid; Glycyrrhetic Acid: $C_{30}H_{46}O_4 = 470.69$

General laboratory reagent grade of commerce consisting of a mixture of α - and β -isomers with the β -isomer predominating.

White to brownish yellow powder; mp, about 292°, with decomposition; $[\alpha]_D^{20}$, +145° to +155° (1 per cent w/v in *ethanol*).

Complies with the following test.

HOMOGENEITY — Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*, prepared using a 25 per cent v/v solution of *phosphoric acid*, to coat the plate.

Mobile phase. A mixture of 5 volumes of *methanol* and 95 volumes of *chloroform*.

Test solution. A 0.5 per cent w/v solution of the reagent under examination in the mobile phase

Apply to the plate 5 µl of the solution. Allow the mobile phase to rise 10 cm. Dry the plate, examine under ultra-violet light at 254 nm. The chromatogram obtained shows a dark spot with an Rf value of about 0.3 (β-glycyrrhetinic acid) and a smaller spot with an Rf value of about 0.5 (α-glycyrrhetinic acid). Spray with *anisaldehyde solution* and heat at 105° for 10 minutes. Both spots are bluish and between them a smaller bluish violet spot may be present.

β-Glycyrrhetinic Acid; 3β-Hydroxy-11-oxo-18β, 20β-olean-12-enoic Acid: $C_{30}H_{46}O_4 = 470.69$

General laboratory reagent grade of commerce.

mp, about 293°; $[\alpha]_D^{20}$, about +170° (1 per cent w/v in *chloroform*).

Glyoxal Sodium Bisulphite: $C_2H_4Na_2O_8S_2,H_2O = 284.17$

General laboratory reagent grade of commerce.

White or almost white, amorphous powder.

Guaiphenesin: Of the Indian Pharmacopoeia.

Guanine; 2-Aminopurin-6-one: $C_5H_5N_5O = 151.1$

General laboratory reagent grade of commerce.

Guanidine Hydrochloride Solution:$CH_5N_3,HCl = 95.1$

Crystalline powder.

Helium: $He = 4.003$

Laboratory cylinder grade of commerce containing not less than 99.995 per cent v/v of He.

Heparin: See Heparin Sodium of the Indian Pharmacopoeia.

HEPES; 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid: $C_8H_{18}N_2O_4S = 238.3$

White or almost white powder.

mp, about 236°, with decomposition.

Heptafluorobutyric Acid: $C_4F_7O_2H = 214.04$

General Laboratory reagent grade of commerce.

Heptafluorobutyric Anhydride: $C_8F_{14}O_3 = 410.1$

Use a grade of commerce suitable for derivatisation.

bp, about 108°; n_D^{20}, about 1.287.

Heptane; *n*-Heptane: $C_7H_{16} = 100.20$

General laboratory reagent grade of commerce.

Clear, colourless, volatile, flammable liquid; bp, about 98°; wt. per ml, about 0.69 g.

Heparin Sodium: Of the Indian Pharmacopoeia.

Hexadecanoic Acid; Palmitic Acid: $C_{16}H_{32}O_2 = 256.43$

General laboratory reagent grade of commerce.

White, crystalline scales; mp, about 63°.

Complies with the following test.

HOMOGENEITY — Carry out test B for Identification described in the monograph for Chloramphenicol Palmitate applying to the plate 4 ml of a 0.2 per cent w/v solution in *acetone*. The chromatogram shows only one spot.

Hexamine; Hexamethylenetetramine: $(CH_2)_6N_4 = 140.19$

Analytical reagent grade of commerce.

Colourless crystals or crystalline powder; odourless.

Hexane; *n*-Hexane: $C_6H_{14} = 86.18$

Analytical reagent grade of commerce containing not less than 90 per cent of *n-hexane*.

Colourless, mobile, highly flammable liquid; bp, about 68°; wt. per ml, about 0.674 g.

Hexane UV: Hexane which complies with the following additional test.

TRANSMITTANCE — Not less than 97 per cent between 260 nm and 420 nm, using *water* as the blank.

Hexanitrodiphenylamine; Dipicrylamine: $C_{12}H_5N_7O_{12} = 439.22$

General laboratory reagent grade of commerce.

Yellow crystals moistened for safety with an equal weight of *water*; mp, about 243°, with explosive decomposition.

Histamine Dihydrochloride: $C_5H_9N_3,2HCl = 184.07$

General laboratory reagent grade of commerce.

White, crystalline powder; mp, about 250°.

Histamine Phosphate: Of the Indian Pharmacopoeia.

DL-Histidine Monohydrochloride; Histidine Monohydrochloride: $C_6H_9N_3O_2HCl,H_2O = 209.63$

General reagent grade of commerce.

Colourless crystals or white, crystalline powder; mp, about 250°, with decomposition.

Holmium Oxide: $Ho_2O_3 = 377.86$

General laboratory reagent grade of commerce.

Yellowish powder.

Holmium Perchlorate Solution: Laboratory reagent grade of commerce for the calibration of spectrophotometer. Consists of a 4 per cent w/v solution of *holmium oxide* in *1.4 M perchloric acid*.

Human Albumin: Of the Indian Pharmacopoeia.

Human Normal Immunoglobulin: Of the Indian Pharmacopoeia.

Hyaluronate Solution: Dilute 1 volume of *sodium hyaluronate stock solution* with 1 volume of *phosphate-buffered saline*.

Use on the day of preparation.

Hyaluronidase Solutions, Diluent for: Mix 250 ml of *phosphate-buffered saline* with 250 ml of *water* and, within 2 hours before use, dissolves 330 mg of *hydrolysed gelatin* in the mixture.

Hydrazine-molybdate Reagent: Dissolve 0.1 g of *ammonium molybdate* in 10 ml of *water* containing 1.5 ml of *sulphuric acid*, dilute to about 90 ml with *water* and add 1 ml of a 0.15 per cent w/v solution of *hydrazine sulphate* and sufficient *water* to produce 100 ml.

Hydrazine Reducing Mixture; Reducing Mixture: Grind together 20 mg of *potassium bromide*, 5 g of *sodium chloride* and 0.5 g of *hydrazine sulphate* in the order stated to produce a homogeneous mixture.

Hydrazine Sulphate: $NH_2NH_2,H_2SO_4 = 130.12$

Analytical reagent grade of commerce.

White, crystalline powder; mp, about 254°.

Hydroiodic Acid: HI = 127.91

Analytical reagent grade of commerce.

Contains about 55 per cent w/w of HI; about 7.5 M in strength.

Colourless or almost colourless liquid when freshly prepared; rapidly becomes yellow or brown due to liberation of iodine; bp, about 127°; wt. per ml, about 1.7 g.

Hydrochloric Acid: Of the Indian Pharmacopoeia.

About 11.5 M in strength.

Hydrochloric Acid AsT: Hydrochloric acid, low in arsenic, commercially available.

Hydrochloric Acid, *x* M: Solutions of any molarity xM may be prepared by diluting 85*x* ml of *hydrochloric acid* to 1000 ml with *water*.

Store in containers of polyethylene or other non-reacting material at a temperature not exceeding 30°.

Hydrochloric Acid AsT, Brominated: Brominated hydrochloric acid low in arsenic, of commercial grade, or prepared by adding 1 ml of *bromine solution* to 100 ml of *hydrochloric acid*.

Hydrochloric Acid, Dilute: Of the Indian Pharmacopoeia. (Approximately 10 per cent w/w of *hydrochloric acid*).

Hydrochloric Acid, Iron-Free: *Hydrochloric acid* which complies with the following additional test.

Evaporate 5 ml on a water-bath nearly to dryness, add 40 ml of *water*, 2 ml of a 20 per cent w/v solution of *citric acid* and 2 drops of *thioglycollic acid*, mix, make alkaline with *dilute ammonia solution* and dilute to 50 ml with *water*; no pink colour is produced.

Hydrochloric Acid, Methanolic: Dilute 4.0 ml of *hydrochloric acid* to 1000.0 ml with *methanol*.

Hydrochloric Acid, *x* M Methanolic: Solutions of any molarity *x*M may be prepared by diluting 85*x* ml of *hydrochloric acid* to 1000 ml with *methanol*.

Hydrochloric Acid AsT, Stannated: Stannated hydrochloric acid, low in arsenic, of commercial grade, or prepared by adding 1 ml of *stannous chloride solution AsT* to 100 ml of *hydrochloric acid AsT*.

Hydrocortisone: Of the Indian Pharmacopoeia.

Hydrogen Cyanide; Hydrocyanic Acid: HCN = 27.03

General laboratory reagent grade of commerce.

Colourless gas or liquid; characterstic odour, very weakly acidic; bp, about 25.6°.

Hydrocyanic Acid Solution: A 0.3 per cent w/v solution of *hydrocyanic acid*. Standardise immediately before use.

ASSAY — To 50 ml in a flask containing 5 ml of *5 M ammonia* add 0.25 ml of *dilute potassium iodide solution* and titrate with *0.1 M silver nitrate* until permanent opalescence is produced.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005405 g of HCN.

Hydrofluoric Acid: HF = 20.01

Analytical reagent grade of commerce.

Colourless, corrosive liquid; odour, pungent; wt. per ml, about 1.13 g; contains not less than 40 per cent w/w of HF.

Store in polyethylene or gutta percha bottles.

Hydrogen Peroxide, $H_2O_2 = 34.01$

ACS reagent grade with an assay content between 29.0 per cent and 32.0 per cent.

Hydrogen Peroxide Solution (100 vol): Of the Indian Pharmacopoeia .

Hydrogen Peroxide Solution (20 vol): Of the Indian Pharmacopoeia.

Hydrogen Peroxide Solution (10 vol); Dilute Hydrogen Peroxide Solution: Dilute *hydrogen peroxide solution (20 vol)* with an equal volume of *water*.

Complies with the following test.

CHLORIDE — To 10 ml add 40 ml of *water*, 1 ml of *dilute nitric acid* and 1 ml of *silver nitrate solution*; no opalescence is produced.

Store as recommended under Hydrogen Peroxide Solution (100 vol).

Hydrogen Sulphide: $H_2S = 34.08$

Use laboratory cylinder grade of commerce or prepare the gas by action of *hydrochloric acid*, diluted with an equal volume of *water*, on *iron sulphide*; wash the resulting gas by passing it through *water*.

Colourless, poisonous gas; odour, characteristic and unpleasant.

Hydrogen Sulphide Solution: A recently prepared saturated solution of *hydrogen sulphide* in *water*.

Hydroquinone; Quinol; 1,4-Dihydroxybenzene; Benzene-1, 4-diol: $C_6H_4(OH)_2 = 110.11$

Analytical reagent grade of commerce.

Colourless or almost colourless crystals or crystalline powder; mp, about 173°.

Store protected from light and moisture.

4-Hydroxybenzaldehyde: $C_7H_6O_2 = 122.12$

General laboratory reagent grade of commerce.

Colourless needles; mp, about 118°.

***p*-Hydroxybenzoic Acid**: $C_7H_6O_3$ = 138.12

Chromatographic grade of commerce.

White crystals; mp, about 216°; contains not less than 97.0 per cent of $C_7H_6O_3$.

ASSAY. Dissolve 0.7 g in 50 ml of *acetone*, add 100 ml of *water*, mix and titrate with *0.5 M sodium hydroxide* determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.5 M sodium hydroxide* is equivalent to 0.06906 g of $C_7H_6O_3$.

4-Hydroxycoumarin: $C_9H_6O_3$ = 162.14

General laboratory reagent grade of commerce.

mp, about 214°.

8-Hydroxy-7-iodoquinoline-5-sulphonic Acid: $C_9H_6INO_4S$ = 351.12

General laboratory reagent grade of commerce.

Yellow, crystalline powder; almost odourless; mp, about 265°, with decomposition.

Hydroxylamine Hydrochloride: Hydroxylammonium Chloride: $NH_2OH.HCl$ = 69.49

Analytical reagent grade of commerce.

Colourless crystals of white, crystalline powder; slowly decomposes when moist; mp, about 151°.

Hydroxylamine Hydrochloride Reagent; Hydroxylamine Hydrochloride Reagent in Ethanol (60 per cent); Hydroxylamine Solution, Ethanolic (60 per cent); Hydroxylamine Solution, Ethanolic: Dissolve 34.75 g of *hydroxylamine hydrochloride* in 950 ml of *ethanol (60 per cent)*, add 5 ml of a 0.2 per cent w/v solution of *methyl orange* in *ethanol (60 per cent)* and then add *0.5 M potassium hydroxide* in *ethanol (60 per cent)* until the full yellow colour is produced. Add sufficient *ethanol (60 per cent)* to produce 1000 ml.

Complies with the following test.

To 10 ml add one drop of *0.5 M potassium hydroxide* in *ethanol (60 per cent)*; no change in colour produced. To a further 10 ml add 1 drop of *0.5 M hydrochloric acid*; the colour changes slightly towards orange.

Hydroxylamine Hydrochloride Solution; Hydroxylamine Solution: Dissolve 1 g of *Hydroxylamine hydrochloride* in 50 ml of *water*, add 50 ml of *ethanol (95 per cent)* and 1 ml of *bromophenol blue solution* and then add *0.1 M sodium hydroxide* until the solution becomes green.

Hydroxylamine Hydrochloride Solution Sp.: Dissolve 20 g of *hydroxylamine hydrochloride* in sufficient *water* to produce about 65 ml. Transfer to a separator, add 5 drops of *thymol blue solution* and *strong ammonia solution* until the solution becomes yellow. Add 10 ml of a 4 per cent w/v solution of *sodium diethyldithiocarbamate* and allow to stand for 5 minutes. Extract with successive quantities, each of 10 ml, of *chloroform* until a 5 ml portion of the extract does not acquire a yellow colour when shaken with *dilute cupric sulphate solution*. Add *dilute hydrochloric acid* until the solution is pink and then with sufficient *water* to produce 100 ml.

Hydroxylamine Solution, Ethanolic (90 per cent): Dissolve 7.0 g of *hydroxylamine hydrochloride* in 90 ml of *ethanol (90 per cent)*, warming gently if necessary, and add 1.6 ml of *dimethyl yellow solution* and sufficient *1 M potassium hydroxide* in *ethanol (90 per cent)* to produce a pure yellow colour. Dilute to 100 ml with *ethanol (90 per cent)*.

***N*-(*p*-Hydroxyphenyl)glycine**; *p*-Hydroxyphenyl-aminoacetic Acid; *p*-Hydroxyanilinoacetic Acid; Photoglycine: $C_8H_9NO_3$ = 167.16

Shiny leaflets from water; browns at 200°; mp, about 246°

8-Hydroxyquinoline: Quinolin-8-ol: C_9H_7NO = 145.16

Analytical reagent grade of commerce.

White to yellowish white, crystalline powder; mp about 74°.

5-Hydroxyuracil; Isobarbituric acid; Pyrimidine-2,4,5-triol; 2,4,5-Trihydroxypyrimidine: $C_4H_4N_2O_3$ = 128.10

General laboratory reagent grade of commerce.

mp, about 310°, with decomposition.

Hyoscine Hydrobromide: Of the Indian pharmacopoeia.

Hyoscyamine Sulphate: $(C_{17}H_{23}NO_3)_2.H_2SO_4.2H_2O$ = 712.82

General laboratory reagent grade of commerce.

mp, about 206°; $[\alpha]_D^{20}$, about -29° (2 per centw/v in *water*).

Hypophosphorus Acid: H_3PO_2 = 65.99

General laboratory reagent grade of commerce.

The water-free acid forms deliquescent crystals; supercools to a colourless, odourless, oily liquid; mp, about 26.5°.

Hypophosphorus Acid, Dilute: General laboratory reagent grade *hypophosphorus acid* of commerce diluted with *water* to contain about 10 per cent w/v of H_3PO_2.

Colourless liquid; wt. per ml, about 1.038 g.

Hypophosphorus Reagent: Dissolve, by heating gently, 10 g of *sodium hypophosphite* in 20 ml of *water* and dilute to 100 ml with *hydrochloric acid*. Allow to settle and decant or filter through glass wool.

Hypoxanthine: $C_5H_4N_4O$ = 136.11

Analytical reagent grade of commerce.

A white to yellowish white powder. Soluble in *1 M sodium hydroxide*.

Iodoethane; Ethyl Iodide: $C_2H_5I = 155.97$

General laboratory reagent grade of commerce.

Heavy, colourless liquid with ethereal odour, turning rapidly to yellowish brown; bp, about 72°, wt. per ml, about 1.94 g.

Imidazole; Glyoxaline: $C_3H_4N_2 = 68.08$

Purified grade of commerce.

White, crystalline powder; mp, about 91°.

Imidazole- Mercury Reagent: Dissolve 8.25 g of *recrystallised imidazole* in 60 ml of *water* and add 10 ml of *5 M hydrochloric acid*. Stir the solution magnetically and add, dropwise, 10 ml of 0.27 per cent w/v solution of *mercuric chloride*. If a cloudy solution results, discard and prepare a further solution by adding the mercuric chloride solution more slowly. Adjust the pH to 6.75 to 6.85 with *5 M hydrochloric acid* (about 4 ml is required) and add sufficient *water* to produce 100 ml.

Imidazole, Recrystallised: Recrystallise 25 g of *imidazole* twice from 100 ml of *toluene*, cooling in an ice-bath with stirring. Finally wash with *ether* and dry at room temperature at a pressure of 2kPa over *silica gel*, or use a purified grade of commerce.

Complies with the following test.

LIGHT ABSORPTION — When examined at about 325 nm (2.4.7), a 8.0 per cent w/v solution shows an absorption of not more than 0.10.

Imidazole Solution: Dissolve 8.25 g of *recrystallise imidazole* in 60 ml of *water*, adjust the pH to 6.75 to 6.85 with *5 M hydrochloric acid* and add sufficient *water* to produce 100 ml.

The hydrochloric acid used in preparing this reagent must be free from stabilising mercury compounds.

Iminodibenzyl: 10,11-Dihyrdo-5*H*-dibenz[*b,f*]azepine: $C_{14}H_{13}N = 195.26$

General laboratory reagent grade of commerce.

Pale yellow, crystalline powder, mp, about 106°.

Imipramine Hydrochloride: Of the Indian Pharmacopoeia.

Immunoglobulin, Normal Human: Of the Indian pharmacopoeia.

Indigo Carmine: Cl 73015; Sodium Indigotindisulphonate; Acid Blue 74: $C_{16}H_8N_2Na_2O_8S_2 = 466.40$

General laboratory reagent grade of commerce.

Deep blue powder or blue granules with a coppery lustre.

Indigo Carmine Solution: To a mixture of 10 ml of *hydrochloric acid* and 990 ml of a 20 per cent w/v solution of *nitrogen-free sulphuric acid* in *water*, add sufficient *indigo carmine* (about 0.2 g) to produce a solution that complies with the following test.

Add 10 ml to a solution of 1.0 mg of *potassium nitrate* in 10 ml of *water*, rapidly add 20 ml of *nitrogen-free sulphuric acid* and heat to boiling; the blue colour is discharged within 1 minute.

Industrial Methylated Spirit: Of the Indian Pharmacopoeia.

Inosine: $C_{10}H_{12}N_4O_5 = 268.23$

White, crystalline powder, mp, about 90° .

Intestinal fluid, simulated: Dissolve 6.8 g of *monobasic potassium phosphate* in 250 ml of *water*, add 77 ml of *0.2 M sodium hydroxide*, 500 ml of *water*, and add 10 g of *pancreatin*, mix and adjusted to pH 6.8 with *0.2 M sodium hydroxide* or *0.2 M hydrochloric acid* and dilute to 1000 ml with *water*.

Iodine: Of the Indian Pharmacopeia.

Iodine, *x* M: Solutions of any molarity x M may be prepared in the following manner. Dissolve 400 x g of *potassium iodide* in the minimum amount of *water*, add 260 x g of *iodine*, allow to dissolve and add sufficient *water* to produce 1000 ml. Weaker solutions may be prepared using proportionately lesser amounts of reagents or by appropriate dilution.

Iodine Bromide: IBr = 206.81

General laboratory reagent grade of commerce.

Bluish or brownish black crystals with lachrymatory vapours; mp, about 40° ; bp, about 116°.

Store protected from light in a cool place.

CAUTION — Iodine bromide vapours are corrosive to the eyes and mucous membranes.

Iodine bromide solution: Dissolve 20 g of *iodine bromide* in sufficient *glacial acetic acid* to produce 1000 ml.

Store protected from light and moisture.

Iodine Monochloride solution: Dissolve 8 g of *iodine trichloride* in about 200 ml of *glacial acetic acid* and separately dissolve 9 g of *iodine* in 300 ml of *dichloromethane*. Mix the two solutions and dilute to 1000 ml with *glacial acetic acid*.

Store in stoppered, light-resistant glass containers at a temperature not exceeding 15°.

Iodine Pentoxide; Recrystallised Iodine Pentoxide: $I_2O_5 = 333.84$

General laboratory reagent grade of commerce recrystallised in the following manner. Boil a saturated solution of commercial iodine pentoxide in *nitric acid* for 1 hour and allow to stand for 24 hours. Decant the supernatant liquid and dry the crystals first in a current of air at room temperature and then over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa.

White or greyish white, crystalline and hygroscopic powder; contains not less than 99.5 per cent of I_2O_5.

ASSAY — Weigh accurately about 0.1 g, dissolve in 50 ml of *water*, add 3 g of *potassium iodide* and 10 ml of *dilute hydrochloric acid* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.002782 g of I_2O_5.

Store protected from light.

Iodine Solution: Dissolve 2.0 g of *iodine* and 3 g of *potassium iodide* in *water* to produce 100 ml.

Iodine Trichloride: $ICl_3 = 233.26$

Analytical reagent grade of commerce.

Reddish orange crystals; pungent irritating odour; volatile at room temperature; mp. about 33°.

Store in tightly-closed, light resistant glass containers preferably in a cool place.

CAUTION — Iodine Trichloride is corrosive to human skin.

Iodoplatinate Reagent: To 3 ml of a 10 per cent w/v solution of *chloroplatinic acid* add 97 ml of *water* and 100 ml of a 6 per cent w/v solution of *potassium iodide*.

Store protected from light.

Iron. Fe = 55.85

Grey powder or wire, soluble in dilute mineral acids.

Iron Salicylate Solution: Dissolve 0.1 g of *ferric ammonium sulphate* in 100 ml of *water* containing 2 ml of *1 M sulphuric acid*, add 50 ml of a 1.15 per cent w/v solution of *sodium salicylate*, 10 ml of *2 M acetic acid* and 80 ml of a 13.6 per cent w/v solution of *sodium acetate* and dilute to 500 ml with *water*.

The solution should be freshly prepared and stored in tightly-closed, light resistant containers.

Isobutyl Acetate: $C_6H_{12}O_2 = 116.16$

General laboratory reagent grade of commerce.

Colourless liquid with a fruity odour; bp, about 118°; refractive index at 20°, about 1.391; wt. per ml, about 0.870 g

Isobutyl alcohol: 2-Methylpropanol; 2-Methyl-1-propanol; 2-Methytlpropan-1-ol: $(CH_3)_2CHCH_2OH = 74.12$

Analytical reagent grade of commerce.

Colourless liquid; odour, characteristic; bp, about 108°; wt. per ml, about 0.801 g.

Isoniazid: Of the Indian Pharmacopoeia.

Isoniazid Solution: Dissolve 0.1 g of *isoniazid* in 150 ml of *methanol* and add 0.12 ml of *hydrochloric acid* and sufficient *methanol* to produce 200 ml.

Isopropylamine; 2-Aminopropane; 2-Propylamine: $CH_3CH(NH_2)CH_3 = 59.11$

Colourless, volatile, flammable liquid having a strong odour of ammonia; bp, about 32°; refractive index at 20°, 1.374 to 1.376; wt. per ml, about 0.69 g.

Isopropyl Myristate: Isopropyl Tetradecanoate with variable amounts of other fatty acid. isopropyl esters; $C_{17}H_{34}O_2 = 270.45$

Clear, practically colourless oily liquid; odourless; refractive index at 20°, about 1.434.

Store protected from light and moisture.

Karl Fischer Reagent: Mix in a 750 ml combustion flask 400 ml of *dehydrated methanol* and 80 g of *dehydrated pyridine*. Immerse the flask in ice and bubble dried *sulphur dioxide* slowly through the mixture until its weight has increased by 20 g. Add 45 g of *iodine* and shake until it dissolves. Allow the solution to stand for 24 hours before use. This solution deteriorates gradually; therefore standardise it within 1 hour before use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration. A commercially available, stabilised solution of Karl Fischer reagent may be used.

The reagent and the solutions used in the determination of water by the Karl Fischer method must be kept anhydrous and precautions must be taken throughout to prevent exposure to atmospheric moisture.

Kerosene: Mixture of hydrocarbons, chiefly of the methane series, as available in commerce.

Kieselguhr; Kieselguhr, Acid-washed: A neutral diatomaceous earth, purified by treating with *dilute hydrochloric acid*, washing with *water* and drying.

Kieselguhr for Column Chromatography: Kieselguhr that complies with the following test.

FILTRATION RATE — Use a chromatography column (0.25 m × 10 mm) with a sintered-glass (100) plate and two marks at 0.10 m and 0.20 m above the plate. Place sufficient of the substance under examination in the column to reach the first mark and fill to the second mark with *water*. When the first drops begin to flow from the column, fill to the second mark again with *water* and measure the time required for the first 5 ml to flow from the column. The flow rate is not less than 1 ml per minute and the eluate obtained is colourless.

Kieselguhr G: consists of Kieselguhr treated with *hydrochloric acid* and calcined, to which is added about 15 per cent w/w of *calcium sulphate hemihydrate* ($CaSO_4,½H_2O$).

Fine, greyish white powder, the grey colour becomes more pronounced on triturating the product with *water*. The average particle size is between 10 µm and 40 µm.

Complies with the following tests.

pH — Shake 1 g for 5 minutes with 10 ml of *carbon dioxide-free water*. The pH of the suspension is between 7.0 and 8.0.

SEPARATING POWER — Determine by thin-layer chromatography (2.4.17), preparing plates using a slurry of the reagent under examination with *0.02 M sodium acetate*.

Mobile phase. A mixture of 65 volumes of *ethyl acetate*, 23 volumes of *2-propanol* and 12 volumes of *water*.

Test solution. A 0.01 per cent w/v solutions in *pyridine* of *lactose, sucrose, dextrose, D-fructose, D-galactose*.

Apply separately to the plate 5 ml of each solution. Allow the mobile phase to rise 14 cm. The migration time of the solvent is about 40 minutes. Dry the plate and spray with about 10 ml of *anisaldehyde solution*, again dry at 105° for 10 minutes. The chromatogram shows five well-defined spots without tailing and well separated from each other.

Kieselguhr H: Fine, greyish white powder, the grey colour becomes more pronounced on triturating the product with *water*. The average particle size is between 10 µm and 40 µm.

Complies with the following tests.

pH — Shake 1 g for 5 minutes with 10 ml of *carbon dioxide-free water*. The pH of the suspension is between 6.4 and 8.0.

SEPARATING POWER — Complies with the test for SEPARATING POWER described under Kieselguhr G.

Kovac's Reagent: Dissolve 5 g of *4-dimethylamino-bezaldehyde* in 75 ml of *amyl alcohol* by warming on a water-bath at 50° to 55°, cool and add 25 ml of *hydrochloric acid*.

Kovac's Reagent should be light yellow to light brown in colour, and should be stored in light-resistant containers at a temperature between 2° and 10°.

Lactose: Of the Indian Pharmacopoeia.

Lactophenol: Dissolve 20 g of *phenol* in a mixture of 20 g of *lactic acid*, 40 g of *glycerin* and 20 ml of *water*.

Lanthanum Nitrate: $La(NO_3)_3,6H_2O = 433.02$

Atomic absorption spectroscopic grade of commerce.

Colourless crystals; deliquescent.

Lanthanum Nitrate Solution: A 5 per cent w/v solution of *lanthanum nitrate*.

Lead Acetate; Sugar of Lead: $(CH_3CO_2)_2Pb,3H_2O = 379.33$

Analytical reagent grade of commerce.

Small, white, transparent, monoclinic prisms or heavy, crystalline masses; odour, acetous; taste, sweet and astringent; efflorescent in warm air; becomes basic when heated.

Store protected from moisture.

Lead Acetate Cotton: Immerse absorbent cotton in a mixture of 10 volumes of *lead acetate solution* and 1 volume of *2 M acetic acid*. Drain off the excess of liquid by placing it on several layers of filter paper without squeezing the cotton. Allow to dry at room temperature.

Store protected from moisture.

Lead Acetate Solution: A 10.0 per cent w/v solution of *lead acetate* in *carbon dioxide-free water*.

Lead Dioxide; Lead oxide: $PbO_2 = 239.20$

Analytical reagent grade of commerce.

Dark brown powder, evolves oxygen when heated, first forming Pb_3O_4 and at high temperatures PbO.

Lead Monoxide; Litharge: $PbO = 223.20$

Analytical reagent grade of commerce.

Pale orange, or pale brick red, heavy scales or powder; odourless.

Lead Nitrate; Lead (II) Nitrate: $Pb(NO_3)_2 = 331.21$

Analytical reagent grade of commerce.

Colourless or white crystals or white, crystalline powder.

Lead Nitrate Solution: A 3.3 per cent w/v solution of *lead nitrate* in *water*.

Lead Nitrate Stock Solution: Dissolve 0.1598 g of *lead nitrate* in 100 ml of *water* to which has been added 1 ml of *nitric acid*, then dilute to 1000 ml with *water*.

Store in polyethylene or glass containers free from soluble lead salts.

Lead Subacetate Solution: Dissolve 40.0 g of *lead acetate* in 90 ml of *carbon dioxide-free water*. Adjust the pH to 7.5 with *10 M sodium hydroxide*, centrifuge and use the clear, supernatant solution. The solution remains clear when stored in a well-closed container.

Linoleic Acid; (9Z, 12Z)-Octadeca-9, 12-dienoic acid: $C_{18}H_{32}O_2 = 280.5$

Colourless, oily liquid, refractive index is about 1.470 at 20°.

Linolenic Acid; (9Z, 12Z, 15Z)-Octadeca-9, 12, 15-trienoic acid: $C_{18}H_{30}O_2 = 278.4$

Colourless liquid, soluble in organic solvents; practically insoluble in water, refractive index at 20° about 1.480

***L*-Leucine**: $C_6H_{13}NO_2 = 131.2$

General laboratory reagent grade of commerce.

A white, crystalline powder; $[\alpha]_D^{20}$, about +14.5 (2 per cent w/v in *6 M hydrochloric acid*).

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin layer chromatography*, using *silica gel G* as the coating substance

and a mixture of 75 volumes of *phenol* and 25 volumes of *water* as the mobile phase. Apply to the plate 5 µl of a 0.2 per cent w/v solution of the reagent being examined and expose the plate to the vapours of the mobile phase for 12 hours before development. After removal of the plate, dry it in a current of warm air, spray with a 0.1 per cent w/v solution of *ninhydrin* in *butan-1-ol* saturated with *water* and heat at 100° to 105° for 10 minutes. The chromatogram shows only one spot.

Lithium: Li = 6.94

General laboratory reagent grade of commerce.

A soft metal, the freshly cut surface of which is silvery grey. It reacts violently with *water* yielding hydrogen and a solution of lithium hydroxide. Before use, the paraffin oil in which the metal is supplied should be washed off with *toluene*.

Store under light paraffin or light petroleum.

Lithium and Sodium Molybdotungstophosphate Solution: Dissolve 100 g of *sodium tungstate* and 25 g of *sodium molybdate* in 800 ml of *water* in a 1500 ml flask, add 50 ml of *phosphoric acid* and 100 ml of *hydrochloric acid* and heat under a reflux condenser for 10 hours. Cool, add 150 g of *lithium sulphate*, 50 ml of *water* and 0.25 ml of *bromine* and allow to stand for 2 hours. Remove the excess of bromine by boiling the mixture for 15 minutes without the condenser. Cool, filter and dilute to 1000 ml with *water*.

Store at a temperature not exceeding 4° and use not later than 4 months after preparation. It has a golden yellow colour and must not be used if any trace of green colour is visible.

Lithium Chloride: LiCl = 42.39.

Analytical reagent grade of commerce.

Deliquescent, cubic crystals, granular or crystalline powder; mp, about 613°.

Lithium Hydroxide: $LiOH,H_2O = 41.96$.

Analytical reagent grade of commercne.

Store protected from moisture.

Lithium Perchlorate: $LiClO_4 = 106.39$.

General laboratory reagent grade of commerce.

White crystals; mp, about 236°.

Lithium Perchlorate, 0.1 M: Dissolve 10.64 g of *lithium perchlorate* in sufficient *water* to produce 1000 ml.

Lithium Sulphate: $Li_2SO_4,H_2O = 127.95$

Analytical grade of commerce.

Colourleass crystals or white crystalline powder

Magenta, Basic; CI 42510; Fuchsin, Basic; Rosaniline Hydrochloride: $C_{20}H_{19}N_3,HCl = 337.85$

Dark red powder or green crystals with a metallic lustre; soluble in *ethanol*; freely soluble in *water*; practically insoluble in *ether*.

Complies with the following test.

When used in the preparation of *decolorised magenta solution*, a nearly colourless solution is obtained.

Magenta Reagent, Decolourised; Fuchsin Reagent, Decolorised: To 1 g of *basic magenta* add 100 ml of *water*, heat to 50° and allow to cool, shaking occasionally. Allow to stand for 48 hours, shake and filter. To 4 ml of the filtrate add 6 ml of *hydrochloric acid*, mix and dilute to 100 ml with *water*. Allow to stand for at least 1 hour to ensure maximum fading.

Magenta Solution, Decolourised; Fuchsin Solution Decolorised: Dissolve 1 g of *basic magenta* in 600 ml of *water*, cool in an ice-bath and add 20 g of *sodium sulphite* dissolved in 100 ml of *water*. Cool in an ice-bath and add, slowly with constant stirring, 10 ml of *hydrochloric acid* and dilute with *water* to 1000 ml. If the resulting solution is turbid, it should be filtered and if brown in colour, it should be shaken with sufficient *decolorising charcoal* (0.2 to 0.3 g) to render it colourless and then filtered immediately. Occasionally it is necessary to add 2 to 3 ml of *hydrochloric acid*, followed by shaking, to remove the little residual pink colour. The solution resulting from any of the foregoing modifications should be allowed to stand overnight before use.

Store protected from light.

Magnesium: Mg = 24.31

General laboratory reagent grade of commerce.

Silvery-white ribbon, turnings, wire or grey powder.

Magnesium Acetate: $(CH_3COO)_2Mg,4H_2O = 214.45$

Analytical reagent grade of commerce.

Colourless crystals; deliquescent.

Magnesium Chloride: Of the Indian Pharmacopoeia.

Magnesium Nitrate: $Mg(NO_3)_2,6H_2O = 256.41$

Analytical reagent grade of commerce.

Colourless crystals; deliquescent.

Magnesium Nitrate Solution: Dissolve 17.30 g of *magnesium nitrate* in 5 ml of *water*, warm gently to effect dissolution, and add 80 ml of *ethanol (95per cent)*. Cool and add sufficient *ethanol (95per cent)* to produce 100 ml.

Magnesium Oxide, Heavy: Of the Indian Pharmacopoeia.

Magnesium Oxide, Light: Of the Indian Pharmacopoeia.

Magnesium Perchlorate: $Mg(ClO_4)_2 = 223.23$

Analytical reagent grade of commerce.

White granules, very hygroscopic, granular or flaky powder.

Complies with the following tests.

ACIDITY OR ALKALINITY — Shake 2 g with 20 ml of *carbon dioxide-free water*; not more than 0.1 ml of either *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required for neutralisation.

WATER (2.3.43) — Not more than 14 per cent w/w.

Magnesium Sulphate: Of the Indian Pharmacopoeia.

Magnesium Sulphate Solution, Ammonical: Dissolve 10 g of *magnesium sulphate* and 20 g of *ammonium chloride* in 80 ml of *water* and add 42 ml of *5 M ammonia*. Allow to stand for a few days in a well closed container, decant and filter.

Magnesium Uranyl Acetate Solution: Heat on a *water* bath 3.2 g of *uranyl acetate*, 10 g of *magnesium acetate*, 2 ml of *glacial acetic acid* and 30 ml of *water*. When the solution is complete allow to cool, add 50 ml of *ethanol* and dilute with *water* to 100 ml. Allow to stand for 24 hours and filter.

Maleic acid; (Z)-But-2-ene-1,4-dioic Acid: Of the Indian Pharmacopoeia.

Mandelic Acid: 2-Hydroxy-2-phenylacetic acid; $C_8H_8O_3$ = 152.1

White crystalline flakes, soluble in *water*; mp, 118° to 121°.

Manganese Sulphate; Manganese (II) sulphate: $MnSO_4,H_2O$ = 169.01.

Analytical reagent grade of commerce.

Pale red, slightly efflorescent crystals.

Complies with the following test.

LOSS ON IGNITION (2.4.20) — 10.0 to 12.0 per cent, determined on 1 g at 500°.

Mannitol: Of the Indian Pharmacopoeia.

D- **Mannose**; Mannose: $C_6H_{12}O_6$ = 180.16.

General laboraory reagent grade of commerce.

Colourless crystals or white crystalline powder; mp, about 132°, with decomposition; $[\alpha]_D^{20}$ about +14.2° (20 per cent w/v in *water* containing about 0.05 per cent w/v of NH_3).

Menthol; (±)-*p*-Methane-3-ol: Of the Indian Pharmacopoeia.

Menthyl Acetate: $C_{12}H_{22}O_2$ = 198.31

Analytical reagent grade of commerce.

Colourless liquid with a characteristic odour; wt. per ml, about 0.92 g.

2-Mercaptoethanol: C_2H_6OS = 78.1

A liquid, miscible with water; d_{20}^{20} about 1.116; bp, about 157°.

Mercuric Acetate; Mercury (II) Acetate: $(CH_3COO)_2Hg$ = 318.68.

General laboratory reagent grade of commerce.

White Crystals or crystalline powder; odour, slightly acetic.

Mercuric Acetate Solution: A 5 per cent w/v solution of *mercuric acetate* in *glacial acetic acid*.

Mercuric Bromide; Mercury (II) Bromide: $HgBr_2$ = 360.40

Analytical reagent grade of commerce.

White crystals or crystalline powder.

Mercuric Bromide Solution, Ethanolic: Dissolve 5 g *mercuric bromide* in 100 ml of *ethanol*, employing gentle heat to facilitate solution.

Store protected from light.

Mercuric Chloride; Mercury (II) Chloride: $HgCl_2$ = 271.50

Analytical reagent grade of commerce.

Heavy, colourless or white, crystalline masses or crystalline powder.

Mercuric Chloride, 0.2 M: Dissolve 54.30 g of *mercuric chloride* in 250 ml of *dilute hydrochloric acid* and make the volume to 1000 ml with *water*.

Mercuric Chloride Solution: A 5 per cent w/v solution of *mercuric chloride*.

Mercuric Nitrate; Mercury (II) Nitrate; $Hg(NO_3)_2,H_2O$ = 342.62.

Analytical reagent grade of commerce.

White or slightly yellow, deliquescent, crystalline powder with odour of nitric acid.

Store protected from light and moisture.

Mercuric Nitrate Solution; Millon's Reagent: Dissolve 3 ml of *mercury* in 27 ml of cold fuming *nitric acid* and dilute the solution with an equal volume of *water*.

Store protected from light and use within 2 months after preparation.

Mercuric Oxide, Nitrogen-free: Yellow mercuric oxide complies with the following additional test, but does not necessarily comply with the requirement for the content of HgO.

NITROGEN — Carry out the determination of nitrogen (2.3.30), using 0.3 g of the sample, 3 g of *anhydrous sodium sulphate*, 0.1 g of *dextrose* and 7 ml of *nitrogen-free sulphuric acid*. The difference between the titrations is not more than 0.1 ml of *0.05 M sulphuric acid*.

Mercuric Oxide, Yellow; Yellow Mercury(II) Oxide: HgO = 216.59

General laboratory reagent grade of commerce.

Orange-yellow, heavy, amorphous powder; odourless; stable in air but becomes discoloured on exposure to light.

Store protected from light and moisture.

Mercuric Sulphate; Mercury(II) Sulphate: $HgSO_4 = 296.68$

General laboratory reagent grade of commerce.

White, odourless granules or crystalline powder; hydrolyses in *water*.

Mercuric Sulphate Solution: Mix 5 g of *yellow mercuric oxide* with 40 ml of *water*, add while stirring, 20 ml of *sulphuric acid* and 40 ml of *water*, and continue stirring until completely dissolved.

Mercury: Hg = 200.59

Analytical reagent grade of commerce.

Shining, Silvery-white, heavy liquid, easily divisible into globules and extremely mobile; readily volatilises on heating; wt. per ml, about 13.5 g.

Metaphosphoric Acid: $(HPO_3)_x$

General laboratory reagent grade of commerce.

Glassy lumps or sticks containing a proportion of sodium metaphosphate; hygroscopic.

Complies with the following tests.

NITRATE — Boil 1.0 g with 10 ml of *water*, cool, add 1 ml of *indigo carmine solution* and 10 ml of *nitrogen-free sulphuric acid* and heat to boiling; the blue colour is not entirely discharged.

REDUCING SUBSTANCES — Boil 1 g with 10 ml of *water* and add 0.1 ml of *0.1 M potassium permanganate*; the pink colour is not entirely discharged.

Metaphosphoric-Acetic Acid Solution: Dissolve 15 g of *metaphosphoric acid* in 40 ml of *glacial acetic acid* and add *water* to produce 500 ml.

Store at a temperature in a cool place and use within 2 days.

Methanesulphonic Acid: $CH_4O_3S = 96.10$

General laboraory reagent grade of commerce.

Colourless, corrosive liquid; wt. per ml, about 1.48g.

Methanesulphonic Acid, 2 M Methanolic: Dissolve 192.2 g of *methanesulphonic acid* in sufficient *methanol* to produce 1000 ml.

Methanol; Methyl Alcohol: $CH_3OH = 32.04$

Analytical reagent grade of commerce.

Clear, clourless liquid; odour, characteristic; bp, about 65°; wt. per ml, about 0.791 g.

When '*methanol*' is followed by a percentage figure, an instruction to use *methanol* diluted with *water* to produce the specified percentage v/v of *methanol* is implied.

Methanol, Acidified: Mix 18 ml of *glacial acetic acid* and 900 ml of *methanol* and dilute to 1000 ml with *water*.

Methanol, Aldehyde-free: Methanol containing not more than 0.001 per cent of aldehydes and ketones. Dissolve 25 g of *iodine* in 1000 ml of *methanol*. Add this solution, with constant stirring to 400 ml of *1 M sodium hydroxide* and add 150 ml of *water*. Allow to stand for 16 hours, filter and boil under a reflux condenser until the odour of iodoform is no longer detectable. Distil the resulting solution by fractional distillation.

Methanol, Anhydrous: Methanol of commerce specially dried for use in Karl Fischer determinations and other non-aqueous titrations.

Complies with the following test.

WATER (2.3.43) — Not more than 0.03 per cent w/v.

Methanol, Dehydrated: *Methanol* which complies with the following additional test.

WATER (2.3.43) — Not more than 0.1 per cent w/w.

Methenamine: $C_6H_{12}N_4 = 140.2$

2-Methoxyethanol; Methoxyethanol; Ethylene Glycol Monomethyl Ether: $C_3H_8O_2 = 76.10$

Chromatographic reagent grade of commerce.

Colourless liquid; bp, about 125°; wt. per ml, about 0.96 g.

Methyl Acetate: $C_3H_6O_2 = 74.1$

Clear colourless liquid soluble in *water*, miscible with *ethanol (95 per cent)*, d_{20}^{20}, about 0.933; n_D^{20}, about 1.361; bp, 56° to 58°.

Methoxyphenylacetic Acid $C_9H_{10}O_3 = 166.2$

General reagent grade of commerce.

Melting point, about 70°.

Store in a cool place.

Methoxyphenylacetic Acid Reagent. Dissolve 2.7 g of *methoxyphenylacetic acid* in 6 ml of *tetramethylammoniumhydroxide solution* and add 20 ml of *ethanol* .

Store in a polyethylene container.

4-Methylaminophenol Sulphate; Methylaminophenol Sulphate; Metol: $(CH_3NHC_6H_4OH)_2,H_2SO_4 = 344.39$

General laboratory reagent grade of commerce.

White or cream coloured, crystalline powder becoming darker on exposure to air.

Methylaminophenol-Sulphite Reagent: Dissolve 0.1 g of *4-methylaminophenol sulphate*, 20 g of *sodium metabisulphite* and 0.5 g of *anhydrous sodium sulphite* in sufficient *water* to produce 100 ml.

Methylaminophenol with Sulphite Solution: Dissolve 0.1 g of *4-methylaminophenol sulphate*, 20 g of *sodium metabi-*

sulphite and 1 g of *sodium sulphite* in sufficient *water* to produce 100 ml.

Methylamphetamine Hydrochloride; Methamphetamine hydrochloride; $C_{10}H_{16}ClN = 185.7$

General laboratory reagent grade of commerce.

3-Methylbenzothiazolin-2-one Hydrazone Hydrochloride: $C_8H_9N_3S,HCl,H_2O = 233.72$

General laboratory reagent grade of commerce.

mp, about 270°.

When used for the determination of aldehydes, complies with the following test.

To 2 ml of *aldehyde-free methanol* add 60 ml of a 0.1 per cent w/v solution of *propionaldehyde* in *aldehyde-free methanol* and 5 ml of a 0.4 per cent w/v solution of the reagent under examination, mix and allow to stand for 30 minutes. Add 25 ml of a 0.2 per cent w/v solution of *ferric chloride hexahydrate*, dilute to 100 ml with *acetone* and mix. The absorbance of the resulting solution at about 660 nm is not less than 0.62. Use as the blank a solution prepared at the same time and in the same manner but omitting the propionaldehyde solution.

Methylcellulose: Of the Indian Pharmacopoeia.

Methylcellulose (4000 cps): Methylcellulose which complies with the following additional test.

VISCOSITY — Between 3000 and 5000 centipoises, determined by method B (2.4.28), on a solution prepared in the following manner. Place 2.0 g, calculated with reference to the dried substance, in a wide-mouthed bottle, add 100 ml of *water* heated to about 90°, close the bottle with a stopper filtered with a stirrer and stir for 10 minutes. Place the bottle in an ice-bath and continue the stirring until a solution of uniform consistency is obtained. Remove from the ice-bath and allow the solution to attain room temperature. Express the results as dynamic viscosity in centipoises.

Methyl *n*-Decanoate; Methyl Caprate: $C_{11}H_{22}O_2 = 186.29$

General laboratory reagent grade of commerce.

Colourless liquid; refractive index at 20°. about 1.425; wt. per ml, about 0.87 g.

Complies with the following additional test.

RELATED SUBSTANCES — Determine by gas chromatography (2.4.13).

Test solution. A 0.002 per cent w/v of the substance under examination in *carbon disulphide*.

Referece solution. A 0.2 per cent w/v of the reagent under examination in *carbon disulphide*.

Chromatographic system

- a glass column 1.5 m x 4 mm packed with silanised diatomaceous support (Diatomite CQ is suitable) (100 to 120 mesh) coated with 10 per cent w/w of silicone gum rubber (methyl) (SE-30 is suitable),
- temperature: 150° and used in conjunction with a precolumn containing silanised glass wool.

The sum of the areas of any secondary peaks in the chromatogram obtained with the tets solution is not greater than the area of the principal peak in the chromatogram obtained with reference solution .

***N*-Methylglucamine**; Meglumine: $C_7H_{17}NO_5 = 195.22$

General laboratory reagent grade of commerce.

mp, about 129°.

Methyl Isobutyrate: $C_5H_{10}O_2 = 102.13$

General laboratory reagent grade of commerce.

Methyl Laurate; Methyl Dodecanoate: $C_{13}H_{26}O_2 = 214.35$

General laboratory reagent grade of commerce containing not less than 98.0 per cent of $C_{13}H_{26}O_2$, determined by gas chromatography (2.4.13).

Colourless or pale yellow liquid; wt. per ml, about 0.87 g.

Methyl Myristate; Methyl Tetradecanoate: $C_{15}H_{30}O_2 = 242.40$

General laboratory reagent grade of commerce containing not less than 98.0 per cent of $C_{15}H_{30}O_2$, determined by gas chromatography (2.4.13).

Colourless or slightly yellow liquid; wt. per ml, about 0.87 g, refractive index at 20° about 1.437, mp about 20°.

Methyl Palmitate; Methyl Hexadecanoate: $C_{17}H_{34}O_2 = 270.46$

General laboratory reagent grade of commerce containing not less than 98.0 per cent of $C_{17}H_{34}O_2$, determined by gas chromatography (2.4.13).

Colourless, waxy solid; fp, about 27°, wt. per ml at 30°, about 0.86 g.

***N*-Methylpiperazine**: $C_5H_{12}N_2 = 100.16$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 138°; wt. per ml, about 0.90 g.

Methyl Salicylate: Of the Indian Pharmacopoeia.

Methyl Stearate: $C_{19}H_{38}O_2 = 298.51$

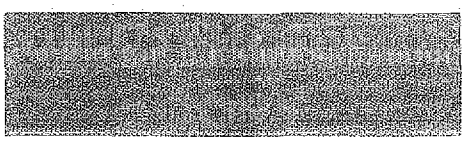
General laboratory reagent grade of commerce containing not less than 98.0 per cent of $C_{19}H_{38}O_2$, determined by gas chromatography (2.4.13).

White or pale yellow, crystalline solid; mp, about 38°.

4,4′-Methylenebis-*N*,*N*-dimethylaniline; Tetramethyldiaminophenylmethane: $C_{17}H_{22}N_2 = 254.37$

General laboratory reagent grade of commerce.

Lustrous leaflets; mp, about 88°.



2-Methyl-5-nitroimidazole: $C_4H_5N_3O_2$ = 127.10

General laboratory reagent grade of commerce.

mp, about 253°.

4-methylpentan-2-one; 4-methyl-2-pentanone; Methyl Isobutyl Ketone: $(CH_3)_2CHCH_2COCH_3$ = 100.16

Analytical reagent grade of commerce

Colourless liquid; bp, about 115°; wt. per ml, about 0.80 g.

2-Methylpropan-2-ol; 2-Methyl-2-propanol; *tert*-butyl Alcohol: $C_4H_{10}O$ = 74.12

Analytical reagent grade of commerce.

Colourless, oily liquid or solid; fp about 25°; bp about 82°; wt. per ml about 0.87 g.

4-Methylpentan-2-ol; 4-Methyl-2-pentanol; $C_6H_{14}O$ = 102.2

Analytical reagent grade of commerce.

Clear, colourless, volatile liquid; bp, about 132°; wt. per ml, about 0.802 g.

Molybdic oxide; Molybdenum(VI) Oxide; Molybdenum Trioxide: MoO_3 = 143.94.

Analytical reagent grade of commerce

White or slightly yellow to slightly bluish powder or granules.

Morphine Hydrochloride: $C_{17}H_{19}NO_3,HCl,3H_2O$ = 374.84

General laboratory reagent grade of commerce.

Colourless, glistening needles or white crystalline powder; $[\alpha]_D^{20}$, about –112° (2 per cent w/v solution); contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{17}H_{19}NO_3,HCl$, calculated with reference to the dried substance.

Myristic Acid: Of the Indian Pharmacopoeia.

Naphthalene: $C_{10}H_8$ = 128.17

Commercial grade suitable for liquid scintillation.

White crystals; mp, about 81°.

1, 3-Naphthalenediol; Naphthalene-1,3-diol; 1, 3-dihdroxy-naphthalene; Naphthorecorcinol: $C_{10}H_8O_2$ = 160.17

General laboratory reagent grade of commerce.

Colourless crystals; mp, about 124°.

Naphthalene-2,7-diol; 2,7-Dihydroxynaphthalene: $C_{10}H_8O_2$ = 160.17

General laboratory reagent grade of commerce.

mp, about 190°, with decomposition.

Naphthalenediol Reagent; Naphthalenediol Solution: Dissolve 10 mg of *naphthalene-2,7-diol* in 100 ml of *sulphuric acid* and allow to stand until the initial yellow colour has disappeared.

Use within 2 days.

Naphthalenediol Reagent Solution: Dissolve 2.5 mg of *naphthalene-2,7-diol* in 90 ml of *methanol* and 10 mg of *potassium ferricyanide* and 50 mg of *potassium cyanide* dissolved in 10 ml of *water*. Allow to stand for 30 minutes and add 100 ml of *0.05 M sodium hydroxide*.

Naphtharson;Thorin;disodium 4-[(2-arsonophenyl)azo]-3-hydroxynaphthalene-2,7-disulphonate $C_{16}H_{11}AsN_2Na_2O_{10}S_2$ = 576.3

Red powder, soluble in *water*.

Naphtharson Solution. A 0.058 per cent w/v solution;

SENSITIVITY — To 50 ml of *ethanol (95per cent)*, add 20 ml of *water* , 1 ml of *0.05M sulphuric acid* and 1 ml of the naphtharson solution. Titrate with *0.025M barium perchlorate* the colour changes from orange-yellow to orange-pink.

Store protected from light; use within one week.

1-Naphthol; α-Naphthol: $C_{10}H_7OH$ = 144.17

Analytical reagent grade of commerce.

Colourless or white crystals or white, crystalline powder; odour, phenolic; mp, about 95°.

Store protected from light.

1-Naphthol Solution: Dissolve 1 g of *1-naphthol* in a solution of 6 g of *sodium hydroxide* and 16 g of *anhydrous sodium carbonate* in 100 ml *water*.

Prepare immediately before use.

1-Naphthol Solution, Dilute: Dissolve 0.1 g of *1-naphthol* in 3 ml of a 15 per cent w/v solution of *sodium hydroxide* and dilute to 100 ml with *water*.

Prepare immediately before use.

2-Naphthol; β-Naphthol: $C_{10}H_7OH$ = 144.17

Analytical reagent grade of commerce.

White leaflets or crystalline powder; odour, faint and resembling that of phenol; mp, about 122°.

Store protected from light.

2-Naphthol Solution: Dissolve 5 g of *2-naphthol*, freshly recrystallised, in 8 ml of *sodium hydroxide solution* and 20 ml of *water* and add sufficient *water* to produce 100 ml.

The solution must be freshly prepared.

1-Naptholbenzein: Naptholbenzein; Phenyl bis(4-hydroxynaphthyl) methanol $C_{27}H_{18}O_2$ = 374.4

Brownish-red powder or shiny brownish-black crystals, practically insoluble in *water*; soluble in *ethanol (95 per cent)* and in *glacial acidic acid*.

1-Naphthylamine; α-Naphthylamine: $C_{10}H_7NH_2 = 143.19$

General laboratory reagent grade of commerce.

White or almost colourless crystals or powder, or crystalline lumps; becoming reddish on exposure to air; odour, unpleasant; mp, about 51°.

Store protected from light.

***N*-(1-Naphthyl)ethylenediamine Dihydrochloride**; *N*-(1-Naphthyl)ethane-1,2-diammonium Dichloride: $NH_2CH_2CH_2NH(C_{10}H_7),2HCl = 259.18$

General laboratory reagent grade of commerce which may contain *methanol* of crystallisation.

White or cream-coloured powder; mp, about 188°.

Nickel Sulphate; Nickel(II) Sulphate: $NiSO_4,7H_2O = 280.9$

Analytical reagent grade of commerce.

Green, crystalline powder or tetragonal crystals.

Ninhydrin; Indane-1,2,3-trione Hydrate: $C_9H_4O_3,H_2O = 178.15$

Analytical reagent grade of commerce.

White or very pale yellow, crystalline powder; mp, about 255°.

Store protected from light.

Ninhydrin and Stannous Chloride Reagent; Ninhydrin-Stannous Chloride Solution: Dissolve 0.2 g of *ninhydrin* in 4 ml of hot *water* and add 5 ml of a 0.16 per cent w/v solution of *stannous chloride*. Allow the mixture to stand for 30 minutes, filter and store at 2° to 8°. Dilute 2.5 ml of the solution with 5 ml of *water* and 45 ml of *2-propanol* immediately before use.

The reagent must be freshly prepared.

Ninhydrin Reagent: Dissolve 4 g of *ninhydrin* in 100 ml of *2-methoxyethanol*, shake gently with 1 g of a cation exchange resin (hydrogen form) (Dowex 50 is suitable) and filter. Add this solution to 100 ml of a 0.16 per cent w/v solution of *stannous chloride* in *acetate buffer pH 5.5*, immediately before use.

The reagent must be freshly prepared.

Ninhydrin Solution: A 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *1-butanol* and 5 volumes of *2 M acetic acid*.

Ninhydrin Solution, Ethanolic: Dissolve 0.1 g of *ninhydrin* in 50 ml of *ethanol (95 per cent)* and add 10 ml of *glacial acetic acid*.

Nitranilic Acid; 2,5-Dihydroxy-3,6-dinitro-1,4-benzoquinone: $C_6H_2N_2O_8 = 230.09$

General laboratory reagent grade of commerce.

Orange or orange-yellow, crystalline powder.

Nitranilic Acid Solution: A 3.5 per cent w/v of *nitranilic acid* in *ethanol (95 per cent)*.

Nitric Acid: $HNO_3 = 63.01$.

Clear, Colourless, fuming liquid; corrosive; about 16 M in strength; wt. per ml, about 1.42 g; contains about 70 per cent w/w of HNO_3.

Store protected from light.

Nitric Acid, *x* M: Solutions of any molarity xM may be prepared by diluting 63x ml of *nitric acid* to 1000 ml with *water*.

Nitric Acid, Dilute: Contains approximately 10 per cent w/w of HNO_3. Dilute 106 ml of *nitric acid* to 1000 ml with *water*.

Nitric Acid, Fuming: $HNO_3 = 63.01$

Analytical reagent grade of commerce.

Clear, almost colourless to yellow, fuming liquid; corrosive; about 22.5 M in strength; wt. per ml, about 1.5 g; contains about 95 per cent w/w of HNO_3.

Store protected from light.

Nitric Oxide: $NO = 30.01$

Nitric oxide, washed with *water*.

4-Nitroaniline; *p*-Nitroaniline: $NO_2C_6H_4NH_2 = 138.13$

Microanalytical reagent grade of commerce.

Deep yellow, crystalline powder; mp, about 148°.

Nitroaniline Solution, Diazotised: Dissolve 0.4 g of *4-nitroaniline* in 60 ml of *1 M hydrochloric acid*, Cool to 15° and add a 10 per cent w/v solution of *sodium nitrite* in *water* until one drop of the mixture turns *starch iodide paper* blue.

Prepare immediately before use.

2-Nitrobenzaldehyde; *o*-Nitrobenzaldehyde: $NO_2C_6H_4CHO = 151.12$

General laboratory grade reagent of commerce.

Yellow needles; odour, almond-like; mp, about 43°.

Nitrobenzene: $C_6H_5NO_2 = 123.11$

Analytical reagent grade of commerce.

Pale yellow liquid; odour, characteristic; bp, about 211°; wt. per ml, about 1.20 g.

4-Nitrobenzoyl Chloride; *p*-Nitrobenzoyl Chloride $NO_2C_6H_4COCl = 185.57$

Analytical reagent grade of commerce.

Yellow crystals or crystalline masses; odour, pungent; decomposes in moist air; mp, about 73°.

4-Nitrobenzyl Bromide; *p*-Nitrobenzyl Bromide: $NO_2C_6H_4CH_2Br = 216.03$

General laboratory reagent grade of commerce.

Pale yellow crystals with a lachrymatory vapour; mp, about 99°.

4-Nitrobenzyl Chloride: *p*-Nitrobenzyl Chloride: $NO_2C_6H_4CH_2Cl = 171.58$

General laboratory reagent grade of commerce.

Pale yellow crystals with a lachrymatory vapour; mp, about 72°.

4-Nitrobenzyl Chloride Solution: A 4.5 per cent w/v solution of *4-nitrobenzyl chloride* in *ethanol*.

4-(4-Nitrobenzyl)pyridine: $C_{12}H_{10}N_2O_2 = 214.22$

General laboratory reagent grade of commerce.

Pale yellow, crystalline powder; mp about 70°.

3-Nitrosalicylic Acid; 2-Hydroxy-3-nitrobenzoic acid: $C_7H_5NO_5 = 183$

General laboratory reagent grade of commerce.

5-Nitrosalicylic Acid; 2-Hydroxy-5-nitrobenzoic acid: $C_7H_5NO_5 = 183$

General laboratory reagent grade of commerce.

Nitrogen: $N_2 = 28.01$

Laboratory cylinder grade of commerce, washed with *water* and dried.

Nitrogen for Chromatography: Nitrogen containing not less than 99.95 per cent v/v of N_2.

Nitrogen, Oxygen-free: Nitrogen that has been freed from oxygen by passage through *alkaline pyrogallol solution*.

Nitromethane: $CH_3NO_2 = 61.04$

General laboratory reagent grade of commerce.

Colourless, oily liquid; odour, strong and somewhat disagreeable; bp, about 101°; wt. per ml, about 1.13 g.

CAUTION — Nitromethane forms explosive compounds with amines and strong bases.

4-Nitrophenyl Disodium Phosphate; 4-Nitrophenyl Disodium Orthopohsphate: $NO_2C_6H_4Na_2PO_4,6H_2O = 371.14$

Analytical reagent grade of commerce.

White crystals.

Complies with the following test.

INORGANIC PHOSPHATE — Dissolve 10 mg in sufficient *water* to produce 100 ml, add 4 ml of *sulphomolybdic acid solution*, shake and add 0.1 ml of *dilute stannous chloride solution*. Allow to stand for 10 minutes. Any blue colour produced is not more intense than that produced in a solution prepared at the same time and in the same manner but using 6.1 ml of *phosphate standard solution (5 ppm PO_4)* in place of the reagent under examination. Compare 20 ml of each solution.

Nitrophenyl Phosphate Solution: Dissolve 4.08 g of *4-nitrophenyl disodium phosphate* in sufficient *diethanolamine buffer pH 10.0* to produce 100 ml.

Store at 4° and use within 24 hours.

Nitroso R Salt; 1-Nitroso-2-naphthol-3,6-disulphonic Acid Disodium Salt: $C_{10}H_5NNa_2O_8S_2 = 377.27$

General laboratory reagent grade of commerce.

Yellow powder.

Nitrous Oxide: Of the Indian Pharmacopoeia.

Nitro-vanado-molybdic:

Solution A. Dissolve 10 g of *ammonium molybdate* in *water*, add 1 ml of *ammonia* and dilute to 100 ml with *water*.

Solution B. Dissolve 2.5 g of *ammonium vanadate* in hot *water*, add 14 ml of *nitric acid* and dilute to 500 ml with *water*.

To 96 ml of *nitric acid* add 100 ml of solution A and 100 ml of solution B and dilute to 500 ml with *water*.

Nonadecanoic Acid: $C_{19}H_{38}O_2 = 298.50$

General laboratory reagent grade of commerce.

mp, about 69°.

Nonan-5-one; Di-*n*-butyl Ketone: $CH_3(CH_2)_3CO(CH_2)_3CH_3 = 142.24$

Analytical reagent grade of commerce.

bp, about 188°; wt. per ml, about 0.83 g.

Noradrenaline Acid Tartrate: Of the Indian Pharmacopoeia.

Normal Serum Reagent: Collect normal human blood in a dry, sterile glass bottle, incubate at 37° for 3 hours, maintain at 4° overnight, remove the serum, dry from the frozen state and keep in a vacuum desiccator over *phosphorus pentoxide*. Dissolve a quantity of the dried serum calculated to have been obtained from 1 ml of the serum in sufficient *imidazole buffer pH 7.4* to produce 10 ml and allow to stand at 4° for 24 to 36 hours.

Noscapine Hydrochloride: $C_{22}H_{23}NO_7,HCl,H_2O = 467.91$

General laboratory reagent grade of commerce.

Colourless crystals or white, crystalline powder; mp, about 200°, with decomposition; hygroscopic; contains not less than 98.5 per cent and not more than 100.5 per cent of $C_{22}H_{23}NO_7$, HCl,H_2O calculated with reference to the dried substance.

Octanoic Acid; Caprylic Acid: $C_8H_{16}O_2 = 144.21$

General laboratory reagent grade of commerce.

Colourless, oily liquid; bp, about 237°; wt. per ml, about 0.92 g.

2-Octanol; Octan-2-ol; *sec*-Octyl Alcohol: $C_8H_{18}O = 130.23$

General laboratory reagent grade of commerce.

Oily liquid ; bp, about 178°; wt. per ml, about 0.82 g.

Octoxinol; Polyoxyethylene (10) isooctylocyclohexyl ether; octylphenoxypolyethoxyethanol; Octoxylenol 9; Polyethylene Glycol Mono-(p-isooctylphenyl) ether: $C_{34}H_{62}O_{11}$ (average) = 646.86 (average).

Clear, pale yellow, viscous liquid; odour faint; hydroxyl value, between 85 and 101.

Octoxinol 10; α-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-ω-hydroxypoly (oxyethylene):

General laboratory reagent grade of commerce (Triton X-100 is suitable).

Clear, pale yellow, viscous liquid.

Store protected from moisture.

Octylamine: *n*-Octylamine; 1-Amino-octane: $C_8H_{19}N = 129.3$

General reagent grade of commerce.

(4-*tert*-Octylphenoxy)nonaethoxyethanol: (*p-tert*-octyl-phenoxy)nonaethoxyethanol; $C_{34}H_{62}O_{11}$ = 646.86

General laboratory reagent grade of commerce.

Oestradiol Benzoate: Of the Indian Pharmacopoeia.

Oleic Acid; (9*Z*)-Octadec-9-enoic acid: Of the Indian Pharmacopoeia

Orcinol; 5-Methylresorcinol: $C_7H_8O_2,H_2O = 142.15$

General laboratory reagent grade of commerce.

White to light tan crystals; mp, about 60°.

Orthophosphorous Acid; Phosphorous acid: $H_3PO_3 = 82.0$

General laboratory reagent grade of commerce.

Osmic Acid; Osmium Tetroxide: $OsO_4 = 254.20$.

General laboratory reagent grade of commerce.

White or light yellow needles or a yellow crystalline mass or very deliquescent crystalline powder; mp, about 40°.

Osmic Acid Solution: General laboratory reagent grade of commerce consisting of a 1 per cent w/v solution of *osmic acid* in *water*.

CAUTION — Osmic acid solution is corrosive to the eyes, mucous membranes and skin.

Oxalic Acid: $(COOH)_2,2H_2O = 126.07$

Analytical reagent grade of commerce.

Colourless Crystals.

Oxalic Acid and Sulphuric Acid; Oxalic Acid- Sulphuric Acid Reagent: A 5 per cent w/v solution of *oxalic acid* in cold *sulphuric acid (50 per cent)*.

Ox Brain, Acetone-Dried: Cut into small pieces a fresh ox brain previously freed from vascular and connective tissue. Place in *acetone* for preliminary dehydration. Complete the dehydration by pounding in a mortar 30 g of this material with successive quantities, each of 75 ml, of *acetone* until a dry powder remains after filtration. Finally dry at 37° for 2 hours until all traces of acetone are removed.

Oxygen: Of the Indian Pharmacopoeia.

Oxytocin: Of the Indian Pharmacopoeia.

Palladium Chloride; Palladous Chloride: $PdCl_2 = 177.33$

General laboratory reagent grade of commerce.

Brownish red powder; hygroscopic; contains not less than 59.3 per cent of Pd.

Palladous Chloride Solution, 0.1 per cent: Dissolve 0.1 g of *palladous chloride* in 100 ml of *0.1 M sodium chloride solution* or 100 ml of *distilled water*.

Papaverine Hydrochloride: $C_{20}H_{21}NO_4HCl = 375.85$

General laboratory reagent grade of commerce.

White or almost white crystals or crystalline powder.

Paracetamol: Of the Indian Pharmacopoeia.

Paracetamol, 4-Aminophenol-free: *Paracetamol* that has been recrystallised from *water* and dried at a pressure of 2 kPa at 70°. If necessary, repeat the procedure until it complies with the following test.

Dissolve 5 g of the dried material in sufficient *methanol (50 per cent)* to produce 100 ml, add 1 ml of freshly prepared *sodium nitroprusside-carbonate solution,* mix and allow to stand for 30 minutes protected from light; no blue or green colour is produced.

Paraffin, Light Liquid: Of the Indian Pharmacopoeia.

Paraffin, Liquid: Of the Indian Pharmacopoeia.

Pararosaniline Hydrochloride; CI 42500; Pararosaniline Chloride; Basic Red 9; $C_{19}H_{18}ClN_3 = 323.82$

General laboratory reagent grade of commerce.

Bluish red, crystalline powder; mp, about 270°, with decomposition.

Pararosaniline Solution, Decolourised: To 0.1g of *pararosaniline hydrochloride* add 60 ml of *water* and a solution of 1.0 g of anhydrous *sodium sulphite* or 2.0 g of

sodium sulphite or 0.75 g of *sodium metabisulphite* in 10 ml of *water*. Add slowly, with stirring, 6 ml of *2 M hydrochloric acid*, stopper the flask and continue stirring until completely dissolve. Dilute to 100 ml with *water*. Allow to stand for 12 hours before use.

Store protected from light.

Penicillinase Solution: Dissolve 10 g of *casein hydrolysate*, 2.72 g of *potassium dihydrogen phosphate* and 5.88 g of *sodium citrate* in 200 ml of *water*, adjust the pH to 7.2 with *sodium hydroxide solution* and dilute to 1000 ml with *water*. Dissolve 0.41 g of *magnesium sulphate* in 5 ml of *water* and add 1ml of a 0.15 per cent w/v solution of *ferrous ammonium sulphate* and sufficient *water* to produce 10 ml. Sterilise both solutions by heating in an autoclave, cool, mix, distribute in shallow layers in conical flasks and inoculate with *Bacillus cereus* (NCTC 9946). Incubate the flasks at 18° to 37° until the growth is observed and then maintain at 35° to 37° for 16 hours, agitating constantly to ensure maximum aeration. Centrifuge and sterilise the supernatant fluid by filtration through a suitable membrane filter.

1.0 ml of Penicillinase Solution will destroy penicillin activity at the rate of at least one million Units per hour at 30° and pH 7.0. Proportionately more is needed to destroy the activity in concentrations of 1000 Units per ml or less. To ensure complete destruction, it is advisable to use 10 times the quantity of penicillinase solution indicated above.

Complies with the tests for sterility (2.2.11).

Store at 0° to 2° and use within 2 to 3 days: When freeze-dried and kept in sealed ampoules, it may be stored for several months.

Pentafluoropropanoic Acid: $C_3HF_5O_2 = 164.0$

Clear, colourless liquid; d_{20}^{20}, about 1.561; n_D^{20}, about 1.284; bp, about 97°.

1-Pentane; *n*-Pentane: $CH_3(CH_2)_3CH_3 = 72.15$

General laboratory reagent grade of commerce.

Clear, colourless, highly flammable, volatile liquid; bp, about 36°; refractive index at 20°, about 1.359; wt. per ml, about 0.63 g.

1-Pentane intended for use in spectrophotometry complies with the following additional test.

TRANSMITTANCE — Not less than 20 per cent at about 200 nm, 50 per cent at about 210 nm, 85 per cent at about 220 nm, 93 per cent at about 230 nm and 98 per cent at about 240 nm, determined using *water* as the blank.

1-Pentanol; Pentan-1-ol; *n*-Pentyl Alcohol: $C_5H_{12}O = 88.15$

General laboratory reagent grade of commerce.

Colourless liquid; bp, about 137°; wt. per ml, about 0.81g.

Pepsin: Of the Indian Pharmacopoeia.

Perchloric Acid: $HClO_4 = 100.46$

Analytical reagent grade of commerce.

A solution in *water* containing between 70 per cent and 72 per cent w/w of $HClO_4$ and about 12 M in strength.

Clear, colourless liquid; very corrosive and may deflagrate on contact with oxidisable substances; wt. per ml, about 1.7 g.

Perchloric Acid, x M: Solutions of any molarity xM may be prepared by diluting 82x ml of *perchloric acid* to 1000 ml with *water*.

Perchloric Acid, 60 per cent:

Analytical reagent grade of commerce.

A solution in *water* containing between 60.0 and 62.0 per cent w/w of $HClO_4$ and about 9 M in strength.

Clear Colourless liquid.

Periodic Acid Reagent: Dissolve 0.5 g of *sodium periodate* in 5 ml of *water*, add 1 ml of *2 M sulphuric acid* and dilute to 10 ml with *water*.

Prepare immediately before use.

Periodic-Acetic Acids Solution: Dissolve 0.446 g of *sodium periodate* in 2.5 ml of *sulphuric acid (25 per cent)* and dilute with *glacial acetic acid* to 100 ml.

Peroxide Test Strips

Use commercial test strips with a suitable scale in the range from 0 ppm to 25 ppm peroxide.

Peroxyacetic Acid Solution: Dilute 1 ml of *hydrogen peroxide (100 vol)* to 100 ml with *glacial acetic acid*. Mix and allow to stand for 12 hours before use.

Use within 24 hours after preparation.

Petroleum, Light; Petroleum Spirit; Petroleum Ether: Analytical reagent grade of commerce.

Colourless, very volatile, highly flammable liquids obtained from petroleum, consisting of a mixture of the lower members of the paraffin series of hydrocarbons and having wt. per ml indicated against each.

Boiling range	Wt. per ml (approx.) (g)
30° to 40°	0.63
40° to 60°	0.64
50° to 70°	0.66
60° to 80°	0.67
80° to 100°	0.70
100° to 120°	0.72
120° to 160°	0.75

Phenacetin; *p*-Ethoxyacetanilide: $C_{10}H_{13}NO_2 = 179.22$

General laboratory reagent grade of commerce.

White, glistening crystalline scales or crystalline powder; odourless; mp, about 135°.

1,10-Phenanthroline; *o*-phenanthroline: $C_{12}H_8N_2,H_2O$ = 198.22

Analytical reagent grade of commerce.

White to cream-coloured crystals or crystalline powder; mp, about 100°.

Phenanthroline Solution: Dissolve 1.5 g of *1,10-phenanthroline* in sufficient *water* to produce 100 ml.

Phenazone; 2,3-Dimethyl-1-phenyl-3-pyrazolin-5-one: $C_{11}H_{12}N_2O = 188.23$

General laboratory reagent grade of commerce.

Colourless crystalline powder; mp, about 112°.

Phenobarbitone: Of the Indian Pharmacopoeia.

Phenol: Of the Indian Pharmacopoeia.

Phenol, Liquified: General laboratory reagent grade of commerce consisting of a solution of *phenol* in *water* containing about 80 per cent w/w of C_6H_6O. Colourless liquid which may acquire a pinkish blue colour on keeping; odour, characteristic and somewhat aromatic; caustic; wt. per ml, about 1.055 g.

Phenol Reagent; Folin and Ciocalteu Phenol Reagent: Readymade reagent commercially available or may be prepared in the following manner.

Dissolve 100 g of *sodium tungstate* and 25 g of *sodium molybdate* in 800 ml of *water* in a 1500 ml flask, add 50 ml of *phosphoric acid* and 100 ml of *hydrochloric acid* and reflux for 10 hours, cool, add 150 g of *lithium sulphate*, 50 ml of *water*, add 4 to 6 drops of *bromine* and allow to stand for 2 hours. Boil for 15 minutes, cool, filter. The reagent should have no greenish tint. Protect from dust. Dilute a portion with an equal volume of *water* before use.

Phenol Solution: A saturated solution of phenol in *water*.

Phenol Saline Solution: A solution containing 0.5 per cent w/v of *phenol*, 0.85 per cent of *sodium chloride* and 2 per cent of *sodium hydroxide* in *water*.

Phenoldisulphonic Acid Solution: A clear liquid which may develop a pale brown colour on storage, prepared either by heating 3 g of *phenol* with 20 ml of *sulphuric acid* on a water-bath for 6 hours and transferring the resulting liquid to a stoppered vessel, or by diluting a 25 per cent w/v solution of commerce with *sulphuric acid* to contain 15 per cent w/v of phenol. The solution complies with the following test.

SENSITIVITY TO NITRATE — Evaporate a solution containing 0.1 mg of *potassium nitrate* to dryness in a porcelain dish on a water-bath. To the cooled residue add 1 ml of the reagent and allow to stand for 10 minutes. Add 10 ml of *water*, cool, add 10 ml of *5 M ammonia* and dilute to 25 ml with *water*. A distinct yellow colour is produced when compared with a solution prepared in the same manner but omitting the potassium nitrate.

Phenoxyacetic Acid; 2-Phenoxyethanoic Acid: $C_6H_5OCH_2COOH = 152.14$

General laboratory reagent grade of commerce.

White or almost white crystals; mp, about 98°.

Complies with the following test.

HOMOGENEITY — Examine under the conditions and at the concentration prescribed in the test for Phenoxyacetic acid described in the monograph for Phenoxymethylpenicillin Potassium applying to the plate 10 µl of a 0.01 per cent w/v solution in *methanol (50 per cent)*. The chromatogram shows only one spot.

Phenoxybenzamine Hydrochloride; *(RS)*-(2-Benzyl-chloroethyl)-1-methyl-2-phenoxyethylamine Hydrochloride: $C_{18}H_{22}ClNO,HCl = 340.29$

General laboratory reagent grade of commerce.

White or almost white crystalline powder, odourless or almost odourless; mp, about 139°.

2-Phenoxyethanol; Phenoxyethanol: $C_8H_{10}O_2 = 138.17$

General laboratory reagent grade of commerce.

Clear, colourless, slightly viscous oily liquid; refractive index at 20°, about 1.537; fp, not less than 12°; wt. per ml, about 1.11 g.

***L*-Phenylalanine**: $C_9H_{11}NO_2 = 165.2$

General laboratory reagent grade of commerce.

A white, crystalline powder; mp, about 272°, with decomposition; $[\alpha]_D^{20}$, about -34 (2 per cent w/v in *water*).

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin layer chromatography*, protected from light, using *silica gel G* as the coating substance and a mixture of 25 volumes of *water* and 75 volumes of *phenol* as the mobile phase, but allowing the solvent front to ascend 12 cm above the line of application. Apply to the plate 5 µl of a 0.2 percent w/v solution and keep the plate in the vapour of the mobile phase for 12 hours before development. After removal of the plate, dry it, spray with a 0.1 per cent w/v solution of *ninhydrin* in *butan-1-ol* saturated with *water* and heat at 100° to 105° for 10 minutes. The chromatogram shows only one principal spot.

(*E*)-4-Phenyl-3-but-3-en-2-one; (*E*)-4-Phenylbut-3-en-2-one; Benzalacetone: $C_{10}H_{10}O = 146.19$

General laboratory reagent grade of commerce.

Lustrous plates; coumarin type odour; mp, about 41°.

4-Phenylenediamine Dihydrochloride; *p*-Phenylenediammonium Dichloride: $C_6H_8N_2,2HCl = 181.08$

General laboratory reagent grade of commerce.

White to pale tan crystals, or crystalline powder, turning pink on exposure to air.

Store protected from light and moisture.

Phenylhydrazine: $C_6H_5NHNH_2 = 108.14$

General laboratory reagent grade of commerce.

Colourless or yellowish liquid, turning yellow or dark red on exposure to light and air; fp, not less than 18°; bp, about 241°, with some decomposition.

Store protected from light and moisture. It should be freshly distilled under reduced pressure before use.

Phenylhydrazine Hydrochloride; Phenylhydrazinium Chloride: $C_6H_5NHNH_2,HCl = 144.60$

Analytical reagent grade of commerce.

White or almost white, crystalline powder, turning brown on exposure to air particularly if exposed to bright light; mp, about 245°, with decomposition.

Store protected from light.

Phenylhydrazine Solution: Phenylhydrazine Hydrochloride Solution; Phenylhydrazine-Sulphuric Acid Solution; Dissolve 65 mg of *phenylhydrazine hydrochloride*, previously recrystallised from *ethanol (85 per cent)*, in sufficient of a mixture of 170 ml of *sulphuric acid* and 80 ml of *water* to produce 250 ml.

Prepare immediately before use.

Phenylmethyl Silicone Fliud (50 per cent Phenyl): Chromatographic reagent grade of commerce.

Phloroglucinol; Benzene-1,3,5-triol; 1,3,5-Trihydroxybenzene: $C_6H_3(OH)_3,2H_2O = 162.14$

Analytical reagent grade of commerce.

White or yellowish crystals or crystalline powder; mp, about 220°.

Phosphatase Enzyme, Alkaline: A suitable enzyme reagent grade of commerce.

Phosphatase Solution, Alkaline: Transfer 3.1 g of *boric acid* to a 1000-ml volumetric flask containing 500 ml of *water*, add 21 ml of *1 M sodium hydroxide* and 10 ml of a 2.0 per cent w/v solution of *magnesium chloride*, dilute to volume with *water* and mix. Adjust the pH of the solution to 9.0 ± 0.2 by addition of *1 M sodium hydroxide* or *1 M hydrochloric acid*, as necessary (solution A).

Dissolve 0.1 g of *alkaline phosphatase enzyme* in 40 ml of solution A in a 50 ml volumetric flask, dilute to volume with solution A and mix. Prepare the solution fresh daily.

Phospholipid: Wash a quantity of normal human or bovine brain freed from meninges and blood vessels and macerate in a suitable blender. Weigh 1000 to 1300 g of the macerate and measure its volume (V ml). Extract with three quantities, each of 4V ml of *acetone*, filter by suction and dry the precipitate at 37° for 18 hours. Extract the dry precipitate with 2 quantities, each of 2V ml, of the mixture of 2 volumes of *light petroleum (boiling range 30° to 40°)* and 3 volumes of *light petroleum (boiling range 40° to 60°)*, filtering each extract through a filter paper previously washed with the light petroleum mixture. Combine the extracts and evaporate to dryness at 45° at a pressure not exceeding 0.7 kPa. Dissolve the residue in 0.2V ml of *ether* and allow to stand at 4° until a deposit is produced. Centrifuge and evaporate the clear supernatant liquid under reduced pressure until the volume is reduced to about 100 ml per kg of the original macerate. Allow to stand at 4° until a precipitate is produced (12 to 24 hours) and centrifuge. To the clear supernatant liquid add 5 volumes of *acetone*, centrifuge, discard the supernatant liquid, dry the precipitate and store protected from light in a vacuum desiccator.

Phosphomolybdic Acid: Dodecamolybdophosphoric Acid: Approximately $H_3PO_4,12MoO_3,24H_2O = 2257.65$

Analytical reagent grade of commerce.

Fine, orange-yellow crystals.

Phosphomolybdic Acid Reagent; Phosphomolybdic Reagent: Dissolve 2.5 g of *phosphomolybdic acid* in sufficient *glacial acetic acid* to produce 50 ml, add 2.5 ml of *sulphuric acid* and mix.

Phosphomolybdic Acid Solution; Phosphomolybdic Acid Spray Solution: A 5.0 per cent w/v solution of *phosphomolybdic acid* in *ethanol*.

Phosphomolybdotungstic Reagent, Dilute: To 1 volume of *phosphomolybdotungstic reagent* add 2 volumes of *water*.

Phosphoric Acid: Of the Indian Pharmacopoeia.

Phosphoric Acid, x M: Solutions of any molarity x M may be prepared by diluting 98x g of *phosphoric acid* to 1000 ml with *water*.

Phosphoric Acid, Dilute: Contains approximately 10 per cent w/v of phosphoric acid. Dilute 69 ml of *phosphoric acid* to 1000 ml with *water*.

Phosphorous Acid: $H_3PO_3 = 82.0$

White or almost white, very hygroscopic and deliquescent crystalline mass; slowly oxidised by air to H_3PO_4.

Unstable, orthorhombic crystals, soluble in *water*, in *ethanol (95 per cent)* and in a mixture of 3 volumes of *ether* and 1 volume of *ethanol (95 per cent)*; melting point, about 73°.

Phosphorus Pentoxide; Diphosphorus Pentoxide: P_2O_5 = 141.95

Grade specially supplied for use in desiccators.

White, amorphous, deliquescent powder; hydrated by *water* with evolution of heat.

Phosphotungstic Acid: Approximately $24WO_3,2H_3PO_4,48H_2O$ = 6625.08

General laboratory reagent grade of commerce.

White or yellowish green crystals or crystalline powder.

Phosphotungstic Acid Solution: Dissolve 10 g of *sodium tungstate* in 75 ml of *water* and add 8 ml of *phosphoric acid*. Heat under a reflux condenser for 3 hours, allow to cool, filter and add sufficient *water* to produce 100 ml.

Phthalaldehyde: $C_8H_6O_2$ = 134.1

General laboratory reagent grade of commerce.

mp about 55°.

Phthalaldehyde Reagent: Dissolve 2.47 g of *boric acid* in 75 ml of *water*, adjust the pH to 10.4 with a 45 per cent w/v solution of *potassium hydroxide* and add sufficient *water* to produce 100 ml. Dissolve 1.0 g of *phthalaldehyde* in 5 ml of *methanol*, add 95 ml of the *boric acid solution* and 2 ml of *thioglycollic acid* and adjust the pH to 10.4 with the potassium hydroxide solution.

Store protected from light and use within 3 days of preparation.

Phthalazine; 2,3-Benzodiazine; Benzo[*d*]pyridazine; α-Phenodiazine: $C_8H_6N_2$ = 130.14

General laboratory reagent grade of commerce.

Pale yellow needles from ether; mp, about 91°; bp, about 316°.

Phthalic Acid; Benzene-1,2-dicarboxylic acid: $C_8H_6O_4$ = 166.1

General laboratory reagent grade of commerce.

A white, crystalline powder.

Phthalic Anhydride: $C_8H_4O_3$ = 148.12

Analytical reagent grade of commerce.

Phthalic Anhydride Solution: Dissolve 42 g of *phthalic anhydride* in 300 ml of *anhydrous pyridine*. Allow to stand for 16 hours.

Store protected from light; use within 1 week.

Picric Acid: 2,4,6-Trinitrophenol: $C_6H_3N_3O_7$ = 229.11

Analytical reagent grade of commerce.

Bright yellow, crystalline powder or yellow prisms or plates moistened with an equal weight of *water* for safety; odourless; very bitter; explodes when heated rapidly or subjected to percussion; mp, about 122°.

CAUTION — When taking mp, the operator should be protected by a glass screen.

Picric Acid Solution; Trinitrophenol Solution: A 1.0 per cent w/v solution of *picric acid* in hot *water*.

Picric Acid Solution, Alkaline; Alkaline Trinitrophenol Solution; Sodium Picrate Solution, Alkaline: Mix 20 ml of *picric acid solution* with 10 ml of dilute *sodium hydroxide solution* and sufficient *water* to produce 100 ml.

The solution should be freshly prepared.

Picrolonic Acid; 3-Methyl-4-nitro-1-(4-nitrophenyl)-5-pyrazolone: $C_{10}H_8N_4O_5$ = 264.2

General laboratory reagent grade of commerce.

Yellow or brownish yellow, crystalline powder; mp, about 116°.

Complies with the following additional test.

SENSITIVITY — Dissolve 25 mg in 10 ml of warm *water* containing 0.1 ml of *glacial acetic acid*. To 1 ml of this solution add 1 ml of a 0.05 per cent w/v solution of *calcium chloride* previously heated to 60°; a bulky precipitate is produced within 5 minutes.

Piperazine Hydrate: Of the Indian Pharmacopoeia.

Plasma, Citrated Rabbit: Collect blood by intracardiac puncture from a rabbit that has been fasted for 12 hours prior to the collection, using a plastic syringe with a No. 1 needle containing a suitable volume of a 3.8 per cent w/v solution of *sodium citrate* so that the final volume ratio of citrate solution to blood is 1: 9. Separate the plasma by centrifugation at 1500 to 1800 rpm at 15° to 20° for 30 minutes.

Store at 0° to 6° and use within 4 hours of collection.

Plasma Substrate; Substrate Plasma: Separate the plasma from 9 volumes of human or bovine blood collected in 1 volume of a 3.8 per cent w/v solution of *sodium citrate* or from 3.5 volumes of human or bovine blood collected in 1 volume of a solution containing 2 per cent w/v of *disodium hydrogen citrate* and 2.5 per cent w/v of *d-glucose*. In the former case, prepare the substrate plasma on the day of collection of blood. In the latter case, the substrate plasma may be prepared upto 2 days after collection of the blood, Store at -20°.

Plasma Substrate Deficient in Factor V: Preferably use congenitally deficient plasma or, alternatively prepare in the following manner; Separate the plasma from human blood collected in 0.1 of its volume of a 1.34 per cent w/v solution of *sodium oxalate* and incubate at 37° for 24 to 36 hours. This plasma should have a clotting time, when tested by the assay method given under *coagulation factor V solution*, of 70 to 100 seconds; if the clotting time is less than 70 seconds, incubate the plasma for a further 12 to 24 hours, Store in small amounts at -20° or below.

Plaster of Paris: Of the Indian Pharmacopoeia.

Poloxamer 188: Poloxamers of the Indian Pharmacopoeia.

Poly(cyanopropyl)siloxane: Polysiloxane substituted with 100 per cent of cyanopropyl groups.

Gas chromatographic reagent grade of commerce.

Polyethylene Glycol 300; Macrogol 300: General laboratory reagent grade of commerce.

Clear, colourless or almost colourless, viscous liquid; wt. per ml, about 1.13 g; refractive index at 20°, about 1.465; viscosity, 71 centistokes at 25°.

Polyethylene Glycol 400; Macrogol 400: General laboratory reagent grade of commerce.

Clear, colourless or almost colourless, viscous liquid; wt. per ml, about 1.13 g; fp, about 6°; viscosity, about 7.1 centistokes at 100°.

Polyethylene Glycol 600; Macrogol 600: General laboratory reagent grade of commerce.

Clear, practically colourless, viscous liquid; odour, slightly characteristic; slightly hygroscopic; viscosity, between 9.9 and 11.1 centistokes at 100°.

Polyethylene Glycol 1000; Macrogol 1000: General laboratory reagent grade of commerce.

Colourless or white, solid mass with a waxy appearance; fp, about 35°; viscosity, about 17.3 centistokes at 100°.

Polyethylene Glycol 20,000; Macrogol 20,000: Chromatographic reagent grade of commerce.

A hard, white, waxy solid; soluble in water with gel formation.

Polyethylene Glycol 20,000 2-Nitroterephthalate; Macrogol 20,000 2-Nitroterephthalate: General laboratory reagent grade of commerce.

White or almost white, hard, waxy solid.

Polyoxyethylene 23 Lauryl Ether; Brij 35: $C_{58}H_{120}O_{24}$ = 1199.6

General laboratory reagent grade of commerce.

mp, about 43°; bp, about 100°.

Polysorbate 20: Of the Indian Pharmacopoeia.

Polysorbate 80: Of the Indian Pharmacopoeia.

Potassium Acetate: CH_3COOK = 98.14

General laboratory reagent grade of commerce.

White powder, or granules or white crystals; odourless or having a faint acetous odour; taste, saline and slightly alkaline; very deliquescent.

Potassium Antimonate; Potassium Pyroantimonate: $KSbO_3,3H_2O$ = 262.90

Analytical reagent grade of commerce.

White, crystalline powder.

Potassium Antimonate Solution: Boil 2 g of *potassium antimonate* with 95 ml of *water* until dissolved. Cool rapidly and add 50 ml of *potassium hydroxide solution* and 1 ml of *2 M sodium hydroxide*. Allow to stand for 24 hours, filter and add sufficient *water* to produce 150 ml.

Use freshly prepared solution.

Potassium Arsenite, 0.1 M: Dissolve 14.60 g of potassium arsenite in sufficient *water* to produce 1000 ml.

Potassium Bicarbonate; Potassium hydrogen Carbonate: $KHCO_3$ = 100.12

Analytical reagent grade of commerce.

Colourless, transparent, monoclinic prisms, or white, granular powder.

Potassium Bicarbonate Solution, Saturated Methanolic: Dissolve 0.1 g of *potassium bicarbonate* in 0.4 ml of *water* by heating on a water-bath. Add 25 ml of *methanol* and swirl, keeping the solution on the water-bath until dissolution is complete.

Prepare immediately before use.

Potassium Bisulphate: Potassium Hydrogen Sulphate; $KHSO_4$ = 136.16

Analytical reagent grade of commerce.

Fused, white lumps, or colourless, transparent, hygroscopic crystals.

Potassium Bromate: $KBrO_3$ = 167.00

Analytical reagent grade of commerce.

White, granular powder or crystals.

Potassium Bromate, 0.0167 M: Dissolve 2.783 g of *potassium bromate* in sufficient *water* to produce 1000 ml.

Potassium Bromate, 0.0333 M: Dissolve 5.566 g of *potassium bromate* in sufficient *water* to produce 1000 ml.

Potassium Bromide: KBr = 119.00

Analytical reagent grade of commerce.

Colourless crystals or white, crystalline powder; odourless.

Potassium Bromide, 0.001 M: Dissolve 0.1190 g of *potassium bromide* in sufficient *water* to produce 1000 ml.

Potassium Bromide IR: Spectroscopic reagent grade complying with the following test.

The infra-red absorption spectrum (2.4.6) of a disc 2 mm thick prepared from the material, previously dried at 250° for 1 hour, has a substantially flat baseline over the range 4000 to 625 cm^{-1}, it exhibits no maxima with an absorbance greater than 0.02 above the base line with the exception of maxima due to *water* at 3440 and 1630 cm^{-1}.

Potassium Carbonate: Anhydrous Potassium carbonate: $K_2CO_3 = 138.21$

Analytical reagent grade of commerce.

White, granular powder; hygroscopic.

Potassium Carbonate, 2 M: Dissolve 276.42 g of *potassium carbonate* in sufficient *water* to produce 1000 ml.

Potassium Carbonate Sesquihydrate: $K_2CO_3,1½H_2O = 165.23$

General laboratory reagent grade of commerce.

Potassium Chlorate: $KClO_3 = 122.55$

Analytical reagent grade of commerce.

White powder, granules or colourless crystals.

CAUTION — In admixture with organic or readily oxidisable substances, it is liable to explode if heated or subjected to percussion trituration.

Potassium Chloride: Of the Indian Pharmacopoeia.

Potassium Chloride IR: Spectroscopic reagent grade complying with the requirement stated under Potassium Bromide IR.

Potassium Chromate: $K_2CrO_4 = 194.19$

Analytical reagent grade of commerce.

Yellow Crystals.

Potassium Chromate Solution: A 5.0 per cent w/v solution of *potassium chromate*.

Potassium Cupri Tartaric Solution, Dilute. Add 1 ml of a solution containing 0.5 per cent w/v of *cupric sulfate* and 1 per cent w/v of *dipotassium tartrate* to 50 ml of *sodium carbonate solution, Alkaline*. Prepare immediately before use.

Potassium Cupri-Tartrate Solution; Alkaline Cupric-Tartrate Solution; Fehling's Solution.

SOLUTION I (COPPER SOLUTION) — Dissolve 34.66 g of carefully selected small crystals of *cupric sulphate*, showing no trace of efflorescence or of adhering moisture, in sufficient *water* to produce 500 ml. Store this solution in small, well stoppered bottles.

SOLUTION II (ALKALINE TARTRATE SOLUTION) — Dissolve 176 g of *sodium potassium tartrate* and 77 g of *sodium hydroxide* in sufficient *water* to produce 500 ml.

Mix equal volumes of the solutions (I) and (II) immediately before use.

Potassium Cyanide: KCN = 65.12

Analytical reagent grade of commerce.

White, crystalline powder or white mass or crystals, gradually decomposing on exposure to air.

CAUTION— Highly poisonous, handle with exteeme care.

Potassium Cyanide Solution: A 10.0 per cent w/v solution of *potassium cyanide*.

Potassium Cyanide Solution Sp.: Dissolve 50 g of *potassium cyanide* in sufficient *water* to produce 100 ml. Remove the lead from this solution by extraction with successive quantities, each of 20 ml, of *dithizone extraction solution* until the *dithizone solution* retains its orange green colour. Extract any dithizone remaining in the cyanide solution by shaking with *chloroform*. Dilute this cyanide solution with sufficient *water* to produce a solution containing 10 g of potassium cyanide in each 100 ml.

Potassium Dichromate: $K_2Cr_2O_7 = 294.18$

Analytical reagent grade of commerce.

Orange-red crystals or crystalline powder.

Potassium dichromate used for the calibration of spectrophotometers contains not less than 99.9 per cent of $K_2Cr_2O_7$, calculated with reference to the substance dried at 130°.

ASSAY — Dissolve 1 g in sufficient *water* to produce 250 ml. Add 50 ml of this solution in a freshly prepared solution of 4 g of *potassium iodide*, 2 g *sodium bicarbonate* and 6 ml of *hydrochloric acid* in 100 ml of *water* in a 500 ml flask. Stopper the flask and allow to stand protected from light for 5 minutes. Titrate with *0.1 M sodium thiosulphate* using 1 ml of *iodide-free starch solution*, added towards the end of the titration, as indicator. 1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.004903 g of $K_2Cr_2O_7$.

Potassium Dichromate Solution: A 10.6 per cent w/v solution of *potassium dichromate*.

Potassium Dichromate Solution, Dilute: A 7.0 per cent w/v solution of *potassium dichromate*.

Potassium Dichromate Solution UV: Dry a quantity of *potassium dichromate* by heating to constant weight at 130°. Weigh accurately a quantity not less than 57.0 mg and not more than 63.0 mg and dissolve in sufficient *0.005 M sulphuric acid* to produce 1000 ml.

Potassium Dihydrogen Citrate; Monobasic Potassium Citrate: $KH_2C_6H_5O_7 = 230.21$

General laboratory reagent grade of commerce.

White, Crystalline powder.

Potassium Dihydrogen Phosphate; Potassium Dihydrogen Orthophosphate; Potassium Phosphate, Monobasic: $KH_2PO_4 = 136.09$

Analytical reagent grade of commerce.

Colourless or white, crystalline powder.

Potassium Dihydrogen Phosphate, x M: Solutions of any molarity xM may be prepared by dissolving 136.09x g of

potassium dihydrogen phosphate in sufficient *water* to produce 1000 ml.

Potassium Ferricyanide; Potassium Hexacyanoferrate(III): $K_3Fe(CN)_6 = 329.25$

Analytical reagent grade of commerce.

Ruby-red crystals.

Potassium Ferricyanide Solution: Wash about 5 g of *potassium ferricyanide* crystals with a little *water* and dissolve the washed crystals in 100 ml of *water*.

Prepare immediately before use.

Potassium Ferricyanide Solution, Dilute: Wash about 1 g of *potassium ferricyanide* crystals with a little *water* and dissolve the washed crystals in 100 ml of *water*.

Produces a blue colour with solutions of iron salts.

Prepare immediately before use.

Potassium Ferrocyanide; Potassium Hexacyanoferrate(II): $K_4Fe(CN)_6,3H_2O = 422.39$

Analytical reagent grade of commerce.

Transparent, yellow crystalline powder.

Potassium Ferrocyanide Solution: A 5.0 per cent w/v solution of *potassium ferrocyanide*.

Potassium Ferrocyanide Solution, Dilute: A 1.0 per cent w/v solution of *potassium ferrocyanide*.

Prepare immediately before use.

Potassium Hydrogen Phthalate: $COOHC_6H_4COOK = 204.22$.

Analytical reagent grade of commerce.

White, crystalline powder.

Potassium Hydrogen Phthalate, x M: Dissolve 204.22x g of *potassium hydrogen phthalate* in about 800 ml of *anhydrous glacial acetic acid*, heat on a water bath until completely dissolved, protected from humidity, cool to 20° and add sufficient *anhydrous glacial acetic acid* to produce 1000 ml.

Potassium Hydrogen (+)-Tartrate: $C_4H_5KO_6 = 188.2$

Analytical reagent grade of commerce.

White, crystalline powder or colourless, slightly opaque crystals.

Potassium Hydroxide; Caustic Potash: $KOH = 56.11$

Analytical reagent grade of commerce.

Contains not less than 85.0 per cent of total alkali calculated as KOH and not more than 2.0 per cent of K_2CO_3.

Dry, white sticks or slabs, pellets or fused mass; hard, brittle and showing a crystalline fracture; very deliquescent; strongly alkaline and corrosive.

Store protected from light and moisture.

Potassium Hydroxide, x M: Solutions of any molarity xM may be prepared by dissolving 56.11x g of *potassium hydroxide* in sufficient *water* to produce 1000 ml.

Potassium Hydroxide Solution, Ethanolic; Alcoholic Potassium Hydroxide: A 10 per cent w/v solution of *potassium hydroxide* in *ethanol*.

Prepare immediately before use.

Potassium Hydroxide Solution, Dilute Ethanolic: Dissolve 3 g of *potassium hydroxide* in 5 ml of *water* and dilute to 100 ml with *aldehyde-free ethanol (95 per cent)*, allow to stand for 1 hour and decant the clear solution.

Prepare immediately before use.

Potassium Hydroxide, x M Ethanolic: Solutions of any molarity x M may be prepared by dissolving 56.11x g of *potassium hydroxide* in sufficient *ethanol (95 per cent)* to produce 1000 ml.

Potassium Hydroxide Solution: A 5 per cent w/v solution of *potassium hydroxide*.

Potassium Iodate: $KIO_3 = 214.00$

Analytical reagent grade of commerce.

White, crystalline powder.

Potassium Iodate, x M: Solutions of any molarity x M may be prepared by dissolving 214.00x g of *potassium iodate* in sufficient *water* to produce 1000 ml.

Potassium Iodate Solution: A 1.0 per cent w/v solution of *potassium iodate*.

Potassium Iodate Solution, 1.5 per cent: Dissolve 1.5 g of *potassium iodate* in 100 ml of 1 per cent w/v *sodium bicarbonate solution*.

Potassium Iodide: Of the Indian Pharmacopoeia.

Potassium Iodide, 1 M: Dissolve 166.00 g of *potassium iodide* in sufficient *water* to produce 1000 ml.

Potassium Iodide and Starch Solution: Dissolve 10 g of *potassium iodide* in sufficient *water* to produce 95 ml and add 5 ml of *starch solution*.

Complies with the following test.

SENSITIVITY TO IODINE — To 15 ml of the solution add 0.05 ml of *glacial acetic acid* and 0.3 ml of *0.0005 M iodine*; a blue colour is produced.

Prepare immediately before use.

Potassium Iodide Solution: A 16.6 per cent w/v solution of *potassium iodide*.

Potassium Iodide Solution, Dilute: A 10 per cent w/v solution of *potassium iodide*.

Potassium Iodide Solution, Iodinated: Dissolve 2 g of *iodine* and 4 g of *potassium iodide* in 10 ml of *water*. When solution is complete, add sufficient *water* to produce 100 ml.

Potassium Iodide Solution, 1.5 per cent: Dissolve 1.5 g of *potassium iodide* in 100 ml of 1 per cent w/v *sodium bicarbonate solution*.

Potassium Iodobismuthate Solution: Dissolve 100 g of *tartaric acid* in 400 ml of *water* and add 8.5 g of *bismuth subnitrate*. Shake during 1 hour, add 200 ml of a 40 per cent w/v solution of *potassium iodide* and shake well. Allow to stand for 24 hours and filter.

Store protected from light.

Potassium Iodobismuthate Solution, Acetic; Potassium Iodobismuthate solution, Acid: Dissolve 8 g of *potassium iodide* in sufficient *water* to produce 20 ml and add the solution to a mixture of 0.85 g of *bismuth subnitrate*, 40 ml of *water* and 10 ml of *glacial acetic acid*.

Potassium Iodobismuthate Solution, Dilute: Dissolve 100 g of *tartaric acid* in 500 ml of *water* and add 50 ml of *potassium iodobismuthate solution*.

Potassium Iodobismuthate Solution, Modified: Suspend 1.7 g of *bismuth subnitrate* and 20 g of *tartaric acid* in 40 ml of *water*. To the suspension add 40 ml of a 40 per cent w/v solution of *potassium iodide*, stir for 1 hour and filter. This stock solution may be kept for several days in light-resistant containers.

Mix 5 ml of the stock solution with 15 ml of *water* immediately before use.

Potassium Iodoplatinate solution: Add 50 ml of a 5 per cent w/v solution of chloroplatinic acid to 45 ml of *potassium iodide solution* and dilute to 100 ml with *water*.

Store in amber-coloured, glass containers.

Potassium Mercuri-Iodide Solution; Mayer's reagent: Add 1.36 g of *mercuric chloride* dissolved in 60 ml of *water* to a solution of 5 g of *potassium iodide* in 20 ml of *water*, mix and add sufficient *water* to produce 100 ml.

Potassium Mercuri-Iodide Solution, Alkaline; Nessler's Reagent: To 3.5 g of *potassium iodide* add 1.25 g of *mercuric chloride* dissolved in 80 ml of *water*, add a cold, saturated solution of *mercuric chloride* in *water*, with constant stirring until a slight red precipitate remains. Dissolve 12 g of *sodium hydroxide* in the solution, add a little more of the cold saturated solution of *mercuric chloride* and sufficient *water* to produce 100 ml. Allow to stand and decant the clear, supernatant liquid.

Potassium monoethyl sulphate: $KC_2H_5SO_4 = 164.19$

Laboratory reagent grade of commerce.

Potassium Nitrate: $KNO_3 = 101.10$

Analytical reagent grade of commerce.

Colourless crystals or white, crystalline powder; odourless; taste, cool and saline.

Potassium Periodate; Potassium meta-Periodate: $KIO_4 = 230.0$

General laboratory reagent grade of commerce.

Potassium Permanganate: Of the Indian Pharmacopoeia.

Potassium Permanganate, x M: Solutions of any molarity x M may be prepared by dissolving 158x g of *potassium permanganate* in 900 ml of *water*, heating on a water-bath for 1 hour, cooling, filtering through a sintered-glass filter and adding sufficient *water* to produce 1000 ml.

Store protected from moisture.

Potassium Permanganate-Phosphoric Acid Solution; Potassium Permanganate and Phosphoric Acid Solution; Potassium Permanganate-Orthophosphoric acid Reagent: Dissolve 3 g of *potassium permanganate* in a mixture of 16 ml of *phosphoric acid* and 70 ml of *water* and add sufficient *water* to produce 100 ml.

Potassium Permanganate Solution; Potassium Permanganate Solution, Strong: A 3.0 per cent w/v solution of *potassium permanganate*.

Potassium Permanganate Solution, Dilute: A 1.0 per cent w/v solution of *potassium permanganate*.

Potassium Perrhenate: $KReO_4 = 289.3$

General laboratory reagent grade of commerce.

Potassium Sulphate; Dipotassium Sulphate: $K_2SO_4 = 174.25$.

Analytical reagent grade of commerce.

White or colourless crystals.

Potassium Tellurite: K_2TeO_3 (approx)

General laboratory reagent grade of commerce.

White granular powder.

Potassium Tetraiodomercurate Solution, Alkaline. Dissolve 11 g of *potassium iodide* and 15 g of *mercury(II) iodide* in *water* and dilute to 100.0 ml with *water*. Immediately before use, mix the solution with an equal volume of a 25 per cent w/v solution of *sodium hydroxide*.

Potassium Tetraoxalate; Potassium Trihydrogen Dioxalate: $KH_3(C_2O_4)_2,2H_2O = 254.19$

Analytical reagent grade of commerce.

White, crystalline powder.

Potassium thiocyanate: $KCNS = 97.18$

Analytical reagent grade of commerce.

Colourless crystals; deliquescent.

Potassium Thiocyanate Solution: A 9.7 per cent w/v solution of *potassium thiocyanate*.

1-Propanol; Propan-1-ol; *n*-Propyl Alcohol; *n*-propanol: $CH_3CH_2CH_2OH = 60.10$

Analytical reagent grade of commerce.

Colourless liquid; bp, about 97°; wt. per ml, about 0.804 g.

2-Propanol; Propan-2-ol; Isopropyl Alcohol; Isopropanol: Of the Indian Pharmacopoeia.

2-propanol intended for use in spectrophotometry complies with the following test.

ABSORBANCE (2.4.1) — Absorbance in the range 320 to 350 nm, measured against *water* as the blank, not more than 0.01 and at about 300 nm, not more than 0.05.

2-Propanol, Anhydrous; Propan-2-ol, Anhydrous: Boil 500 ml of *2-propanol* under a reflux condenser with 5 g of *stannous chloride* for 30 minutes. Test for the absence of peroxides by adding 0.5 ml of the cooled solution to 1 ml of *dilute potassium iodide solution* acidified with 0.5 ml of *2 M hydrochloric acid* and adding 0.1 ml of *starch solution*. The absence of a blue colour after standing for 1 minute indicates the absence of peroxides.

If peroxides are still present, add 2.5 g of *stannous chloride* and boil under a reflux condenser for another 30 minutes. Repeat the procedure until the test shows the absence of peroxides. Add 100 g of *calcium oxide*, boil under a reflux condenser for 4 hours and distil, discarding the first 100 ml and taking adequate precautions to exclude moisture.

Propionaldehyde; Propanal: $C_3H_6O = 58.08$

General laboratory reagent grade of commerce.

Liquid; odour, suffocating; bp, about 49°; refractive index at 20°, about 1.365; wt. per ml, about 0.81 g.

Propylene Glycol: Of the Indian Pharmacopoeia.

Propylene Oxide: $C_3H_6O = 58.1$

General laboratory reagent grade of commerce.

bp, about 34°, refractive index at 20°, about 1.366.

Propyl Gallate: Of the Indian Pharmacopoeia.

Pumice Powder: The particles of pumice of commerce, powdered and sifted, which passes through a *No. 22 sieve*, but are retained by a *No. 60 sieve*.

Pumice Stone: A substance of volcanic origin consisting chiefly of complex silicates of aluminium and alkali metals. Very light, hard, rough, porous, greyish masses; odourless; stable in air.

Purine: $C_5H_4N_4 = 120.11$

White to off-white powder.

MELTING RANGE — Between 214° and 217°.

Putrescine; 1,4-Butanediamine; Tetramethylenediamine: $C_4H_{12}N_2 = 88.15$

Colourless oily liquid, very soluble in water. Strong piperidine-like odour.

bp, about 159°.

mp, about 23°.

Pyridine: $C_5H_5N = 79.10$

Analytical reagent grade of commerce.

Clear, colourless liquid; odour, characteristic and unpleasant; hygroscopic; bp, about 115°; refractive index at 20°, about 1.510; wt. per ml, about 0.98 g.

Pyridine, Anhydrous; Pyridine, Dehydrated: Dry *pyridine* over *anhydrous sodium carbonate*, filter and distil.

Complies with the following additional test.

WATER (2.3.43) — Not more than 0.01 per cent w/w.

Pyridine Bromide Solution: Dissolve 8 g of *pyridine* and 5.4 ml of *sulphuric acid* in 20 ml of *glacial acetic acid*, keeping the mixture cool. Add 2.5 ml of *bromine* dissolved in 20 ml of *glacial acetic acid* and dilute with *glacial acetic acid* to 1000 ml.

Prepare immediately before use.

Pyridine-Acetic Anhydride Reagent: Just before use, add cautiously with cooling 25 ml of freshly distilled *acetic anhydride* to 50 ml of freshly distilled *anhydrous pyridine*, cool and dilute with freshly distilled *anhydrous pyridine* to 100 ml.

Pyrimethamine: Of the Indian Pharmacopoeia.

Pyrogallol; 1,2,3-Trihydroxybenzene: $C_6H_3(OH)_3 = 126.11$

General laboratory reagent grade of commerce.

White or slightly discoloured, light or heavy crystals; mp, about 133°.

Store protected from light.

Pyrogallol Solution, Alkaline: Dissolve 0.5 g of *pyrogallol* in 2 ml of *water*; dissolve 12 g *potassium hydroxide* in 8 ml of *water*. Mix the two solutions immediately before use.

Quinhydrone; Cycohexa-2,5-diene-1,4-dione compound with Benzene-1,4-diol: $C_{12}H_{10}O_4 = 218.21$

Analytical reagent grade of commerce.

Dark green, lustrous crystals or crystalline powder; mp, about 171°.

Quinidine Sulphate: Of the Indian Pharmacopoeia.

Quinine: $C_{20}H_{24}N_2O_2 = 324.4$

General laboratory reagent grade of commerce.

Microcrystalline powder; efflorescent; mp, about 57°, with decomposition; $[\alpha]_D^{20}$, about –167° (1 per cent in *ethanol*).

Store protected from light.

Quinine Sulphate: Of the Indian Pharmacopoeia.

Quinoline: $C_9H_7N = 129.15$

General laboratory reagent grade of commerce, suitable for phosphate assay.

Clear, pale yellow liquid; odour, characteristic and unpleasant; hygroscopic; bp, about 237°; refractive index at 20°, about 1.626; wt. per ml, about 1.095 g.

Complies the following test.

TARRY IMPURITIES — Dissolve 1.0 g in a mixture of 2.5 ml of *water* and 2.5 ml of *hydrochloric acid*. Add 3 ml of *water* and 1 ml of *potassium chromate solution* and heat to boiling; the solution shows no darkening and no black deposit.

Store protected from light.

Quinoline Solution: Dissolve 50 ml of *quinoline* in a mixture of 60 ml of *hydrochloric acid* and 300 ml of *water*, previously heated to 70°, cool and filter.

Rabies Antiserum, Fluorescein-Conjugated: Immunoglobulin fraction with a high rabies antibody titre, prepared from the sera of suitable animals that have been immunised with inactivated rabies virus. The immunoglobulin is conjugated with fluorescein isothiocyanate.

Rabbit Erythrocyte Suspension: Prepare a 1.6 per cent w/v suspension of rabbit erythrocytes in the following manner. Defibrinate 15 ml of freshly drawn rabbit blood by shaking with glass beads, centrifuge at 2000 rpm for 10 minutes and wash the erythrocytes with three quantities, each of 30 ml of *saline solution*. Dilute 1.6 ml of the suspension of erythrocytes to 100 ml with a mixture of 1 volume of *phosphate buffer pH 7.2* and 9 volumes of *saline solution*.

Resorcinol: Resorcin; Benzene-1,3-diol; 1,3-dihydroxybenzene: $C_6H_4(OH)_2 = 110.11$

Analytical reagent grade of commerce.

Colourless or nearly colourless needle-shaped crystals or powder; odour, slight but characteristic; mp, about 111°.

Resorcinol Solution: Shake 0.2 g of *resorcinol* with 100 ml of *toluene* until saturated and decant.

Prepare immediately before use.

Rhodamine B; CI 45170; Basic Violet 10; D & C Red No. 19; Tetraethylrhodamine; *N*-[9-(2-Carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene]-*N*-ethylethanaminium Chloride: $C_{28}H_{31}ClN_2O_3 = 479.02$

General laboratory reagent grade of commerce.

Green crystals or reddish violet powder.

Saccharin Sodium: Of the Indian Pharmacopoeia.

Salicyladehyde; 2-Hydroxybenzaldehyde: $C_7H_6O_2 = 122.12$

General laboratory reagent grade of commerce

Clear, colourless, oily liquid; odour, characteristic; bp, about 196°; refractive index at 20°, about 1.574; wt. per ml, about 1.167 g.

Salicylic Acid: Of the Indian Pharmacopoeia.

Saline Solution: A 0.9 per cent w/v solution of *sodium chloride* in freshly distilled *water*, sterilised by heating in an autoclave (2.2.11).

Saline Solution, Pyrogen-free: *Saline solution* complying with the pyrogens (2.2.8).

Saline Solution, Sterile: Of the Indian Pharmacopoeia.

Semicarbazide Acetate Solution: Triturate 2.5 g of *semicarbazide hydrochloride* with 3.3 g of *sodium acetate*, add 10 ml of *methanol*, mix, transfer to a flask with the aid of 20 ml of *methanol*, allow to stand at a temperature of about 4° for 30 minutes, filter and add sufficient *methanol* to produce 100 ml.

Semicarbazide Hydrochloride: $NH_2CONHNH_2,HCl = 111.53$.

Analytical reagent grade of commerce.

White to yellowish white crystals or crystalline powder; mp, about 176°, with decomposition.

Selenium Dioxide: $SeO_2 = 110.96$

General reagent grade of commerce.

L-Serine: $C_3H_7NO_3 = 105.1$

General laboratory reagent grade of commerce

A white, crystalline solid; mp, about 222°, with decomposition; $[\alpha]_D^{20}$, about +14 (10 per cent in *1 M hydrochloric acid*).

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin layer chromatography*, using *silica gel G* as the coating substance and a mixture of 25 volumes of *water* and 75 volumes of *phenol* as the mobile phase but allowing the mobile phase to ascend 12 cm above the line of application. Apply 5 µl of a 0.2 per cent w/v solution to the plate and keep the plate in the vapours of the mobile phase for 12 hours before development. After removal of the plate, dry it in air, spray with a 0.1 per cent w/v solution of *ninhydrin* in *butan-1-ol* saturated with *water* and heat at 100° to 105° for 10 minutes. The chromatogram shows only one principal spot.

Serum Solution: Dilute 1 volume of *serum stock solution* with 3 volumes of *acetate buffer solution* and adjust to room temperature.

Use on the day of preparation.

Serum Stock Solution: Dilute 1 volume of cattle or horse serum (native or reconstituted with *water* to its original volume) with 9 volumes of *acetate buffer solution*. Adjust the

pH to 3.1 with *4 M hydrochloric acid* and allow to stand for 18 to 24 hours.

Store at 0° to 4° and use within 30 days.

Sesame Oil: Refined fixed oil obtained from the seeds of one or more cultivated varieties of *Sesamum indicum*.

Pale yellow oil; almost odourless; wt. per ml, about 0.92 g.

Silica Gel; Silica Gel for Chromatography: A grade of commerce suitable for column chromatography.

Precipitated silicic acid in form of lustrous granules.

Silica Gel, Anhydrous; Silica Gel, self-indicating: A grade of commerce consisting of partially dehydrated, polymerised, colloidal silicic acid containing cobalt chloride as an indicator.

Blue granules, becoming pink when the moisture adsorption capacity is exhausted. Anhydrous silica gel absorbs about 30 per cent of its wieght of water at 20°. Its adsorptive capacity may be regenerated by heating at 150° for 2 hours.

Silica Gel, Butylsilyl; Butylsilyl Silica Gel for Chromatography: A grade of commerce suitable for column chromatography. A very finely divided Silica Gel (3 to 10 µm) chemically modified at the surface by the introduction of butylsilyl groups. The particle size is indicated after its name in tests where it is specified for use.

Silica Gel F254; Fine, white, homogeneous powder of an average particle size of less than 30 µm containing a fluorescent indicator having a maximum intensity at about 254 nm used for thin-layer chromatography. (Merck silica gel 60 F254 precoated plates are generally satisfactory).

Silica Gel G: Fine, white, homogeneous powder of an average particle size between 10 and 20 µm containing about 13 per cent w/w of *calcium sulphate hemihydrate* ($CaSO_4,½H_2O$).

Complies with the following tests.

CALCIUM SULPHATE CONTENT — Carry out the test described under Aluminium Oxide G.

pH (2.4.24) — About 7, determined in a suspension prepared by shaking 1 g with 10 ml of *carbon dioxide-free water* for 5 minutes.

SEPARATING POWER — Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. Toluene.

Test solution. A solution in *dichloromethane* containing 0.01 per cent w/v of each of *dimethyl yellow*, *indophenol blue* and *sudan red G* in *toluene*.

Apply to the plate 10 µl of the solution. Allow the mobile phase to rise 10 cm. The chromatogram shows three clearly separated spots, the spot due to *indophenol blue* near the starting point, that due to *dimethyl yellow* in the middle of the chromatogram and that due to *sudan red G* between the two.

Silica Gel GF254; Silica Gel G/UV254: Fine, white, homogeneous powder of an average particle size between 10 and 20 µm containing about 13 per cent w/w of *calcium sulphate hemihydrate* ($CaSO_4,½H_2O$) and about 1.5 per cent w/w of a fluorescent indicator having a maximum intensity at about 254 nm.

Complies with the test for CALCIUM SULPHATE CONTENT described under Aluminium Oxide G, with the tests for pH and SEPARATING POWER described under Silica Gel G and with the following test.

FLUORESCENCE — Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *2-propanol* and 10 volumes of *anhydrous formic acid*.

Test solution. A 0.1 per cent w/v solution of *benzoic acid* in the same solvent mixture.

Apply separately to the plate increasing quantities from 1 to 10 µl of the test solution. After development, dry the plate in a current of warm air and examine under ultra-violet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram at levels of 2 µg and greater.

Silica Gel H: Fine, white, homogeneous powder of an average particle size between 10 µm and 20 µm.

Complies with the tests for pH and SEPARATING POWER described under Silica Gel G.

Silica Gel H, Silanised: Fine, white, homogeneous powder which, after shaking with *water*, floats on the surface because of its water-repellent properties. It complies with the test for pH described under Silica Gel G and with the following test.

SEPARATING POWER — Determine by thin-layer chromatography (2.4.17), prepared in the following manner. Vigorously shake 30 g of the silica gel for 2 minutes with 60 ml of a mixture of 2 volumes of *water* and 1 volume of *methanol* and, using a spreading device designed for the purpose, spread a uniform layer of the suspension about 0.25 mm thick on a series of carefully cleaned glass plates measuring 20 cm × 5 cm. Allow the coated plates to dry in air and heat in an oven at 105° to 110° for 30 minutes.

Mobile phase. A mixture of 65 volumes of *dioxan*, 25 volumes of *water* and 10 volumes of *glacial acetic acid*.

Test solution. A mixture of 0.1 g of each of methyl laurate, methyl myristate, methyl palmitate and methyl stearate add 40 ml of a decanted 3 per cent w/v solution of *potassium hydroxide* in *ethanol (90 per cent)* and heat under a reflux condenser on a water-bath for 1 hour. Cool, add 100 ml of *water*, acidify the mixture with *2 M hydrochloric acid* and extract with three quantities, each of 10 ml, of *chloroform*. Dry

the combined chloroform extracts over *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dissolve the residue in 50 ml of *chloroform*.

Apply separately to the plate three quantities, each of 10 ml. After development, dry the plate by heating in an oven at 120° for 30 minutes. Allow to cool and spray with a 3.5 per cent w/v solution of phosphomolybdic acid in *2-propanol* at 150° until the spots become visible. Expose the plate to ammonia vapour until the background turns white; each chromatogram shows 4 clearly separated spots.

Silica Gel HF254; Silica Gel H/UV 254: Fine, white, homogeneous powder of an average particle size between 10 and 20 µm containing about 1.5 per cent w/w of a fluorescent indicator having a maximum intensity at about 254 nm.

Complies with the tests for pH and SEPARATING POWER described under Silica Gel G and with the test for FLUORESCENCE described under Silica Gel GF254.

Silica Gel HF254, Silanised: Fine, white, homogeneous powder which after shaking with *water*, floats on the surface because of the water-repellent properties.

Complies with the test for SEPARATING POWER described under Silanised Silica Gel H.

Silica Gel, Strong Anion-exchange: A strong anion exchange packing for high performance liquid chromatography made by chemically bonding a quaternary amine to a silica spherical core, 10 µm to 50 µm in diameter, the particle size being generally indicated by the number after the name of the reagent, e.g. Partisil 10 SAX.

Siliceous Earth, Chromatographic: For gas chromatography, use a specially prepared grade meeting the following general description.

Purified siliceous earth of suitable mesh size that has been acid and/or base-washed. It may or may not be silanised.

For column partition chromatography, it is essential that the material be free from interfering substances. If such interferences are known or thought to be present, purify the material as follows. Place a pledget of glass wool in the base of a chromatographic column having a diameter of 100 mm or larger and add *purified siliceous earth* to a height equal to 5 times the diameter of the column. Add a volume of *hydrochloric acid* equivalent to one-third the volume of the siliceous earth and allow the acid to percolate into the column. Wash the column with *methanol*, using small volumes at first to rinse the walls of the column, and continue washing with *methanol* until the last washing is neutral to moistened *litmus paper*. Extrude the washed column into shallow dishes, heat on a steam-bath to remove the excess *methanol* and dry at 105° until the material is powdery and free from traces of *methanol*. Store the dried material in well-closed containers.

Siliceous Earth, Purified: Purified siliceous earth is a form of silica (SiO_2) consisting of the frustules and fragments of diatoms purified by calcining.

Store protected from moisture.

Silicon Dioxide: SiO_2,xH_2O

General laboratory reagent grade of commerce.

Fine, white, hygroscopic, odourless, amorphous powder; diameter of the average particles, between 2 µm and 10 µm.

Silicon Dioxide, Anhydrous: $SiO_2 = 60.08$

Analytical reagent grade of commerce.

Transparent crystals.

Silicone Oil: Use Dimethicone or any other silicone oil.

Silicotungstic Acid; Dodecatungstosilicic acid: $H_4SiW_{12}O_{40}$ + aq.

General laboratory reagent grade of commerce.

White or yellowish white crystals; deliquescent.

Store protected from moisture.

Silver Nitrate: Of the Indian Pharmacopoeia.

Silver Nitrate, *x* M: Solutions of any molarity xM may be prepared by dissolving 170x g of *silver nitrate* insufficient *water* to produce 1000 ml.

Store protected from light.

Silver Nitrate Solution: A 5.0 per cent w/v solution of *silver nitrate*.

Store protected from light.

Silver Nitrate Solution, Ammonical; Silver Ammonio-nitrate Solution: Dissolve 2.5 g of *silver nitrate* in 80 ml of *water* and cautiously add *dilute ammonia solution* until the precipitate first formed is nearly dissolved; set aside, decant and add sufficient *water* to produce 100 ml.

Prepare immediately before use.

CAUTION — Dry ammonical silver nitrate is very explosive.

Silver Nitrate Solution, Dilute: A 1.7 per cent w/v solution of *silver nitrate*.

Store protected from light.

Silver Nitrate Solution, Methanolic: Boil, until dissolved, 5 g of *silver nitrate* with 60 ml of *methanol* under reflux on a water-bath. Use the solution while still hot.

Silver Nitrate-Pyridine Reagent; Silver Nitrate Solution in Pyridine: A 5.0 per cent w/v solution of *silver nitrate* in *pyridine*.

Silver Oxide: $Ag_2O = 231.74$.

General laboratory reagent grade of commerce.

Brownish black powder.

Store protected from light and moisture. Do not expose to ammonia fumes or easily oxidisable substances.

Soda Lime: General purpose grade of commerce. White granules, about 4 to 10 mesh (1.7 to 4.0 mm).

Sodium: Na = 22.99

General laboratory reagent grade of commerce.

Soft silvery grey metal.

Store under light petroleum or liquid paraffin.

Sodium Acetate: Of the Indian Pharmacopoeia.

Sodium Acetate, Anhydrous: $CH_3COONa = 82.03$

Analytical reagent grade of commerce.

White, pale-grey or very pale brown, crystalline masses; hygroscopic.

Complies with the following test.

LOSS ON DRYING — Not more than 2 per cent when dried at 105°.

Sodium Acetate Solution, 0.1 M: Dissolve 13.61 g of *sodium acetate* in sufficient *water* to produce 1000 ml.

Sodium Acid Citrate; Disodium Hydrogen Citrate: $C_6H_6Na_2O_7, 1½ H_2O = 263.11$

General laboratory reagent grade of commerce.

White powder; odourless or almost odourless.

Sodium Azide: $NaN_3 = 65.0$

Analytical reagent grade of commerce.

Sodium Arsenite; Sodium Metaarsenite: $NaAsO_2 = 129.91$

General laboratory reagent grade of commerce.

White or greyish white powder; somewhat hygroscopic.

Sodium Arsenite, 0.1 M: Dissolve 9.892 g of *arsenic trioxide* in 80 ml of *1 M sodium hydroxide*, dilute to 800 ml with *water* and add *2 M hydrochloric* acid until the solution is neutral to *litmus paper*. Add 2 g of *sodium bicarbonate*, dissolve by stirring and dilute with *water* to produce 1000 ml.

Sodium Arsenite Solution: Dissolve 0.50 g of *arsenic trioxide* in 5 ml of *2 M sodium hydroxide* and add 2.0 g of *sodium bicarbonate* and sufficient *water* to produce 100 ml.

Sodium Bicarbonate: Of the Indian Pharmacopoeia.

Sodium Bicarbonate, x M: Solutions of any molarity xM may be prepared by dissolving 84.01x g of *sodium bicarbonate* in sufficient *water* to produce 1000 ml.

Sodium Bicarbonate Solution; Sodium Hydrogen Carbonate Solution: A 5 per cent w/v solution of *sodium bicarbonate*.

Sodium Bismuthate: $NaBiO_3 = 280.00$

Analytical reagent grade of commerce containing not less than 85.0 per cent of $NaBiO_3$.

Yellow to yellowish brown powder, slowly decomposing when moist or at a high temperature.

ASSAY — Suspend 0.2 g in 10 ml of a 20 per cent w/v solution of *potassium iodide* and add 20 ml of *1 M sulphuric acid*. Titrate with *0.1 M sodium thiosulphate* using 1ml of *starch solution*, added towards the end of the titration, as indicator until an orange solution is produced. 1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.01400 g of $NaBiO_3$.

Sodium Bisulphite; Sodium Acid Sulphite: $NaHSO_3 = 104.07$

White or yellowish white crystals or granular powder, odour, of sulphur dioxide; unstable in air.

Consists of sodium bisulphite, $NaHSO_3$, and sodium metabisulphite, $Na_2S_2O_5$, in varying proportions and yielding not less than 58.5 per cent w/w and not more than 67.4 per cent w/w of SO_2, determined by the following method.

ASSAY — Weigh accurately about 0.2 g, transfer to a glass-stoppered flask, add 50 ml of *0.05 M iodine* and insert the stopper. Allow to stand for 5 minutes, add 1 ml of *hydrochloric acid* and titrate the excess of iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titriation, as indicator. 1 ml of *0.05 M iodine* is equivalent to 0.003203 g of SO_2.

Store protected from moisture in a cool place.

Sodium Bisulphite Solution: Dissolve 10 g of *sodium bisulphite* in sufficient *water* to produce 30 ml.

Prepare immediately before use.

Sodium Bromide: NaBr = 102.9

Analytical reagent grade of commerce.

Sodium Butanesulphonate; 1-Butanesulphonic Acid Sodium salt: $C_4H_9NaO_3S = 160.17$

Chromatographic grade of commerce.

Sodium Butanesulphonate, x M: Solutions of any molarity x may be prepared by dissolving 160.17 x g of *sodium butanesulphonate* in sufficient *water* to produce 1000 ml.

Sodium Carbonate: $Na_2CO_3,10H_2O = 286.15$

Analytical reagent grade of commerce.

Transparent, colourless, rhombic crystals; odourless; efflorescent.

Sodium Carbonate, Anhydrous: $Na_2CO_3 = 105.99$

Analytical reagent grade of commerce.

White, hygroscopic powder which loses not more than 1 per cent of its weight on heating to about 300°.

Sodium Carbonate, x M: Solutions of any molarity x M may be prepared by dissolving 286.15 x g of *sodium carbonate* in sufficient *water* to produce 1000 ml.

Sodium Carbonate Solution: A 10.6 per cent w/v solution of *anhydrous sodium carbonate.*

Sodium Carbonate Solution, Alkaline: A 2 per cent w/v solution of *sodium carbonate, anhydrous* in 0.1M *sodium hydroxide.*

Sodium Carbonate Solution, Dilute: A 10 per cent w/v solution of *sodium carbonate.*

Sodium Chloride: Of the Indian Pharmacopoeia.

Sodium Chloride Injection: Of the Indian Pharmacopoeia.

Sodium Chloride Solution; Brine: A saturated solution of *sodium chloride.*

Sodium Citrate: Of the Indian Pharmacopoeia.

Sodium Cobaltinitrite; Sodium Hexanitritocobaltate(III): $Na_3Co(NO_2)_6 = 403.94$

Analytical reagent grade of commerce.

Orange-yellow powder.

Sodium Cobaltinitrite Solution: A 30 per cent w/v solution of *sodium cobaltinitrite.*

Sodium 1-Decasulphonate: $C_{10}H_{22}NaO_3S = 245.34$

Chromatographic reagent grade of commerce.

Sodium 1-Decasulphonate Solution: Dissolve 0.24 g of *sodium 1-decasulphonate* in 1000 ml of *water.*

Sodium Diethyldithiocarbamate: $(C_2H_5)_2NCSSNa,3H_20 = 225.30$

Analytical reagent grade of commerce.

White or colourless crystals.

Sodium Diethydithiocarbamate solution: A 0.1 per cent w/v solution of *sodium diethyldithiocarbamate.*

Prepare immediately before use.

Sodium Dihydrogen Phosphate; Sodium Dihydrogen Orthophosphate: Of the Indian Pharmacopoeia.

Sodium Dihydrogen Orthophosphate, Anhydrous; Anhydrous sodium dihydrogen phosphate: $NaH_2PO_4 = 120.0$ Analytical reagent grade of commerce.

Store protected from moisture.

Sodium Dihydrogen Orthophosphate Monohydrate: Sodium dihydrogen phosphate monohydrate; $NaH_2PO_4,H_2O = 138.0$

Analytical reagent grade of commerce.

Store protected from moisture.

Sodium Dihydrogen Phosphate, x M: Solutions of any molarity xM may be prepared by dissolving 156x g of *sodium dihydrogen phosphate* in sufficient *water* to produce 1000 ml.

Sodium Dihydrogen Phosphate Dihydrate: Of the Indian Pharmacopoeia

Sodium Ferrocyanide; Sodium Hexacyanoferrate (II): $Na_4Fe(CN)_610H_20 = 484.06$

General laboratory reagent grade of commerce.

Yellow Crystals or granules.

Soduium Fluoride: Of the Indian Pharmacopoeia.

Sodium Formate: $HCOONa = 68.01$

General laboratory reagent grade of commerce.

White, deliquescent granules or crystalline powder; odour, slightly that of formic acid; mp about 253°.

Store protected from moisture.

Sodium Heptanesulphonate; 1-Heptanesulphonic Acid Sodium salt: $C_7H_{15}NaO_3S = 202.25$

Chromatographic reagent grade containing not less than 96.0 per cent of $C_7H_{15}NaO_3S$.

Sodium Heptanesulphonate, 0.025 M: Dissolve 5.056 g of *sodium heptanesulphonate* in sufficient *water* to produce 1000 ml.

Sodium Heptanesulphonate Monohydrate: $C_7H_{15}NaO_3S,H_2O = 220.26$

Chromatographic reagent grade of commerce containing not less than 96 per cent of $C_7H_{15}NaO_3S$, calculated with reference to the anhydrous substance.

Complies with the following tests.

WATER (2.3.43) — Not more than 8.0 per cent determined on 0.3 g.

ASSAY — Dissolve 0.15 g in 50 ml of *anhydrous glacial acetic acid*, titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02022 g of $C_7H_{15}NaO_3S$.

Sodium Hexanesulphonate; Hexanesulphonic Acid Sodium Salt: $C_6H_{13}NaO_3S = 188.23$.

Chromatographic reagent grade of commerce.

Sodium Hexanesulphonate, 0.03 M: Dissolve 5.65 g of *sodium hexanesulphonate* in sufficient *water* to produce 1000 ml.

Sodium Hyaluronate: Cut human umbilical cords, freed from blood and stored under *acetone*, into segments 2 cm long and wash with 10 volumes of *acetone*. Wash by soaking in three quantities, each of 10 volumes, of *water* for 2, 2 and 24 hours. Mince the cords, add an equal volume of *water*, adjusted to pH 2.0 with *2 M hydrochloric acid* add 3 g of *pepsin* for each kg of cords, cover with a layer of *toluene* and incubate at 37 for 24 hours, maintaining the pH at 2.0. Adjusted to pH 7.4

by the caution addition of a 40 per cent w/v solution of *sodium hydroxide*, add 5 g of *trypsin* for each kg of cords and incubate at 37° for 24 hours. Centrifuge the aqueous layer, decant the clear liquid, cool to 5°, adjust the pH to 2.0 with a mixture of equal volumes of *hydrochloric acid* and *water*, and add with stirring 2 volumes of *ethanol (95 per cent)*. Separate the precipitate by centrifuging, suspend it in 300 ml of *water* for each kg of the cords and dialyse against running tap-water for 24 hours. To each litre for suspension add 660 ml of *chloroform* 340 ml of *amyl alcohol* and 200 ml of a solution containing 30 per cent w/v of *sodium acetate* and 16 per cent w/v of *glacial acetic acid* in *water*. Shake vigorously for 10 minutes, centrifuge and treat the separated aqueous phase repeatedly with further portions of the chloroform-amyl alcohol mixture until no precipitate is formed at the interface. To the aqueous phase add 2 volumes of *ethanol (95 per cent)*, centrifuge dissolve the residue in *water*, dialyse against running tap-water for 24 hours and then against several changes of *water* for 24 hours; dry the product from the frozen state.

Sodium Hyaluronate Stock Solution: Prepare a stock solution to contain in each ml 500 mg of *sodium hyaluronate*, previously dried over *phosphorus pentoxide* under reduce pressure for 48 hours. Store at a temperature below 0° and use within 30 days. Do not keep the sodium hyaluronate over *phosphorus pentoxide* indefinitely.

Sodium Hydroxide: Of the Indian Pharmacopoeia.

Sodium Hydroxide, x M: Solutions of any molarity xM may be prepared by dissolving 40x g of *sodium hydroxide* in sufficient *water* to produce 1000 ml.

Sodium Hydroxide, x M Ethanolic: Solutions of any molarity xM may be prepared by dissolving 40x g of *sodium hydroxide* in sufficient *ethanol (95 per cent)* to produce 1000 ml.

Sodium Hydroxide Solution: A 20.0 per cent w/v solution of *sodium hydroxide*.

Sodium Hydroxide Solution, Dilute: A 5.0 per cent w/v solution of *sodium hydroxide*.

Sodium Hydroxide Solution, Strong: A 42 per cent w/v solution of *sodium hydroxide* (about 10 M).

Sodium Hypobromite Solution: Mix 20 ml of *10 M sodium hydroxide* and 500 ml of *water* in an ice-bath, add 5 ml of *bromine solution* and stir gently until solution is complete. Prepare immediately before use.

Sodium Hypobromite Solution, Alkaline: Dissolve 10 g of *sodium hydroxide* in 400 ml of *water*, add 5.5 ml of *bromine*, stir to dissolve and add sufficient water to produce 500 ml. Ascertain the strength of the solution by adding to 10 ml, 25 ml of *water*, 2 g of *potassium iodide* and 10 ml of *glacial acetic acid* and titrating the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added toward the end of the titration, as indicator. 1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.0080 g of available bromine, Dilute the remainder of the solution to contain 1.5 per cent w/v of available bromine; to 66 ml of the resulting solution add 20 ml of *sodium hydroxide solution* and sufficient *water* to produce 100 ml.

Sodium Hypochlorite Solution: General laboratory reagent grade of commerce containing not less than 10.0 per cent w/v and not more than 14.0 per cent w/v of available chlorine.

Clear, colourless to pale yellow liquid having odour of chlorine. It is affected by light and gradually deteriorates.

ASSAY — Transfer 1 ml in a glass-stoppered flask containing a solution of 3 g of *potassium iodide* in 100 ml of *water*, add 20 ml of *dilute acetic acid* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003546 g of available chlorine.

Store protected from light preferably in a cool place.

Sodium Hypochlorite Solution (3 per cent Cl): Dilute 30 ml of *sodium hypochlorite* solution to 100 ml with *water* immediately before use. The solution contains not less than 2.5 per cent and not more than 3.0 per cent w/v of available chlorine determined by the following method.

ASSAY — Add to a flask, sucessively 50 ml of *water*, 1 g of *potassium iodide* and 12.5 ml of *2 M acetic acid*. Dilute 10 ml of the reagent under examination to 100 ml. with *water*, add 10 ml. of this solution to the flask and titrate with *0.1 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003546 g of available chlorine.

Store protected from light preferably in a cool place.

Sodium Hypochlorite Solution (3.5 per cent Cl): Dilute 35 ml of *sodium hypochlorite solution* to 100 ml with *water* immediately before use.

The solution contains approximately 3.5 per cent w/v of available chlorine determined by the method described under sodium hypochlorite solution (3 per cent Cl).

Sodium Hypophosphite: NaH_2PO_2,H_2O=106.00

General laboratory reagent grade of commerce.

Crystalline powder or colourless crystals; hygroscopic.

Sodium Iodide: NaI = 149.89

Analytical reagent grade of commerce.

White crystals or granules ; deliquescent.

Sodium Lauryl Sulphate; Sodium Dodecyl Sulphate: Of the Indian Pharmacopoeia.

Sodium Lauryl Sulphate, x M: Dissolve 288.4 ×g of *sodium lauryl sulphate*, in sufficient *water* to produce 1000 ml. Prepare immediately before use.

Sodium Metabisulphite: Of the Indian Pharmacopoeia.

Sodium Methanesulphonate; methanesulphonic acid, sodium salt: $CH_3SO_3Na = 118.1$

General laboratory reagent grade of commerce.

Hygroscopic, store protected from moisture.

Sodium Molybdate: $Na_2MoO_4,2H_2O = 242.00$

Analytical reagent grade of commerce.

Crystalline powder.

Sodium Molybdotungstophosphate Solution: Boil 350 ml of *water*, 50 g of *sodium tungstate*, 12 g of *phosphomolybdic acid* and 25 ml of *phosphoric acid* for 2 hours in a flask fitted with a reflux condenser. Cool and add sufficient *water* to produce 500 ml.

Sodium 1,2-Naphthaquinone-4-Sulphonate: $C_{10}H_5NaO_5S = 260.20$

General laboratory reagent grade of commerce.

Yellow or orange, crystalline powder.

Sodium Nitrate: $NaNO_3 = 84.99$

Analytical reagent grade of commerce.

Colourless crystals or white, granular powder; deliquescent in humid air.

Sodium Nitrite: $NaNO_2 = 68.99$

Analytical reagent grade of commerce containing not less than 97.0 per cent of $NaNO_2$, calculated with reference to the substance dried over *anhydrous silica gel* for 4 hours.

Colourless to slightly yellow crystals or white or slightly yellow, granular powder.

Sodium Nitrite Solution: A 10 per cent w/v solution of *sodium nitrite*.

Prepare immediately before use.

Sodium Nitroprusside; Sodium Pentacyanonitrosylferrate (III) Dihydrate: $Na_2[Fe(CN)_5(NO)],2H_2O = 297.95$

Analytical reagent grade of commerce.

Reddish brown powder or crystals.

Sodium Nitroprusside Carbonate Solution; Sodium Nitroprusside Solution, Alkaline: Dissolve 1 g of *sodium nitroprusside* and 1 g of *anhydrous sodium carbonate* in sufficient *water* to produce 100 ml.

Sodium Nitroprusside Solution: A 1.0 per cent w/v solution of *sodium nitroprusside*.

Prepare immediately before use.

Sodium Octanesulphonate; Octanesulphonic Acid Sodium Salt: $C_8H_{17}NaO_3S = 216.27$

General laboratory reagent grade of commerce.

Sodium Octanesulphonate, 0.02 M: Dissolve 4.33 g of *sodium octanesulphonate* in sufficient *water* to produce 1000 ml.

Sodium Octyl Sulphate; Sodium 4-Octyl Sulphate: $C_8H_{17}NaO_4S = 232.27$

Chromatographic reagent grade of commerce.

Sodium Oxalate: $C_2Na_2O_4 = 133.99$

Analytical reagent grade of commerce.

White, crystalline powder.

Sodium Pentanesulphonate; Sodium 1-Pentanesulphonate: $C_5H_{11}NaO_3S = 174.19$

Chromatographic reagent grade of commerce.

Sodium Pentanesulphonate, Monohydrate; 1-Pentanesulphonic acid sodium salt monohydrate: $C_5H_{11}NaO_3S, H_2O = 192.2$

Chromatographic reagent grade of commerce.

Sodium Perchlorate; Sodium Perchlorate Monohydrate: $NaClO_4,H_2O = 140.46$

Analytical reagent grade of commerce.

White, deliquescent crystals.

Sodium Periodate; Sodium Metaperiodate: $NaIO_4 = 213.89$

Analytical reagent grade of commerce containing not less than 99.0 per cent of $NaIO_4$.

Sodium Periodate Solution: Dissolve 1.07 g of *sodium periodate* in *water* and add 5 ml of *1 M sulphuric acid* and sufficient *water* to produce 100 ml.

The solution should be freshly prepared.

Sodium Peroxide: $Na_2O_2 = 77.98$

Analytical reagent grade of commerce.

Yellowish white, granular powder.

Store protect from light and moisture.

CAUTION — Protect from contact with organic or oxidisable substances.

Sodium Phosphate: Of the Indian Pharmacopoeia.

Sodium Phosphate, Anhydrous: Anhydrous Dibasic Sodium Phosphate; Anhydrous Disodium Hydrogen Phosphate; $Na_2HPO_4 = 141.96$.

Analytical reagent grade of commerce.

Sodium Phosphate Solution: Dissolve 12 g of clear crystals of *sodium phosphate* in *water* to make 100 ml.

Sodium Phosphate, Monobasic; Sodium Biphosphate; Sodium Dihydrogen Phosphate; Acid Sodium Phosphate; Monosodium Orthophosphate: $NaH_2PO_4,H_2O = 138.0$

General reagent grade of commerce.

Sodium Phosphate, Monobasic, Dihydrate; Sodium Dihydrogen Phosphate, Dihydrate: $NaH_2PO_4,2H_2O = 156.01$

Use a suitable grade with a content of not less than 99.0 per cent.

Sodium Phosphate, Tribasic; Trisodium Phosphate; Trisodium Orthophosphate: $Na_3PO_4,12H_2O = 380.13$

Analytical reagent grade of commerce.

Colourless or white crystals.

Sodium Potassium Tartrate; Potassium Sodium Tartrate; Potassium sodium (+) -Tartarate: $COONaCHOHCHOHCOOK,4H_2O = 282.17$

Analytical reagent grade of commerce.

Colourless prismatic crystals or white, crystalline powder.

Sodium Pyrophosphate: $Na_4P_2O_7,10H_2O = 446.06$

Analytical reagent grade of commerce.

Colourless,slightly efflorescent crystals.

Sodium Rhodizonate; Rhodizonic acid disodium salt; [(3,4,5,6-Tetraoxocyclohex- 1-en-1,2-ylene)dioxy]disodium: $C_6O_6Na_2 = 214.0$

Violet crystals, soluble in *water* with an orange-yellow colour.

Prepare immediately before use.

Sodium Salicylate: Of the Indian Pharmacopoeia.

Sodium Salicylate Solution: A 10.0 per cent w/v solution of *sodium salicylate*.

Sodium silicate: Na_2SiO_3,xH_2O

Chemically pure grade of commerce.

White powder.

Sodium Sulphate, Anhydrous: $Na_2SO_4 = 142.04$

Analytical reagent grade of commerce.

White crystalline powder or granules; hygroscopic

Complies with the following test.

LOSS ON DRYING — Not more than 0.5 per cent when dried at 130°.

Sodium Sulphide: $Na_2S,9H_2O = 240.18$

Analytical reagent grade of commerce.

Colourless crytals or cyrstalline masses which turns yellow on storage; deliquescent.

Sodium Sulphide Solution: A 10.0 per cent w/v solution of *sodium sulphide*.

Sodium Sulphite: $Na_2SO_37H_2O = 252.15$

General laboratory reagent grade of commerce containing not less than 95.0 per cent of $Na_2SO_3,7H_2O$

White colourless crystals.

Sodium Sulphite, Anhydrous: $Na_2SO_3 = 126.05$

Analytical reagent grade of commerce containing not less than 95 per cent of Na_2SO_3.

Small crystals or powder.

Sodium Tartrate; Sodium(+)-Tartrate: $C_4H_4O_6Na_2,2H_2O = 230.08$

Analytical reagent grade of commerce.

Transparent crystals.

Sodium Thioglycollate; Sodium Mercaptoacetate; Mercaptoacetate Acid Sodium salt: $SHCH_2COONa = 114.09$

General laboratory reagent grade of commerce.

Hygroscopic crystals; odour, slight and characteristic.

Sodium Thiosulphate: Of the Indian Pharmacopoeia.

Sodium Thiosulphate, x M: Solutions of any molarity x M may be prepared by dissolving 248 x g of *sodium thiosulphate* and 2x g of *sodium carbonate* in sufficient *carbon dioxide-free water* to produce 1000 ml.

Sodium Tungstate: $Na_2WO_4,2H_2O = 329.86$

Analytical reagent grade of commerce.

Colourless crystals or white, crystalline powder.

Sorbitol: Of the Indian Pharmacopoeia.

Squalane; 2,6,10,15,19,23-Hexamethyltetracosane: $C_{30}H_{62} = 422.81$

Gas chromatographic reagent grade of commerce.

Colourless oily liquid; refractive index at 20°, 1.451 to 1.453; wt. per ml, 0.811 to 0.813 g.

Standard solution for the determination of water: This solution is prepared, generally in acid conditions, from the element or a salt of the element whose minimum content is not less than 99.0 per cent. The quantity of per litre of solution is more than 0.995 g throughout the period, as long as the vial has not been opened. The starting material (element or salt) and the characteristic of the final solvent (nature and acidity etc) are mentioned on the label.

Stannous Chloride; Tin (II) Chloride: $SnCl_2,2H_2O = 225.63$

Analytical reagent grade of commerce.

Colourless crystals; contains not less than 97.0 per cent of $SnCl_2,2H_2O$.

ASSAY — Dissolve 0.5 g in 15 ml of *hydrochloric acid* in a ground-glass stoppered flask and add 10 ml of *water* and 5 ml of *chloroform*. Titrate rapidly with *0.05 M potassium iodate* until the *chloroform* layer is colourless.

1 ml of *0.05 M potassium iodate* is equivalent to 0.02256 g of $SnCl_2,2H_2O$.

Stannous Chloride Solution: Prepared by either of the following two methods.

(1) Dissolve 330 g of *stannous chloride* in 100 ml of *hydrochloric acid* and add sufficient *water* to produce 1000 ml.

(2) Dilute 60 ml of *hydrochloric acid* with 20 ml of *water*, add 20 g of tin, heat gently until no more gas is evolved and add sufficient *water* to produce 100 ml. Store over a little of the undissolved tin remaining in the solution and protected from air.

Stannous Chloride Solution AsT: Stannous Chloride Solution, low in arsenic, commercially available or prepared from *stannous chloride solution* by adding an equal volume of *hydrochloric acid AsT*, reducing to the original volume by boiling and filtering through a fine-grain filter paper. It complies with the following test.

To 10 ml add 6 ml of *water* and 10 ml of *hydrochloric Acid AsT*, distil and collect 16 ml. To the distillate add 50 ml of *water*, 0.1 ml of the solution, 5 ml of *0.1 M potassium iodide* and 5 g of *zinc AsT*. Use the apparatus and method described in Appendix 2.3.10. The stain produced on *mercuric chloride paper* is not more intense than that produced when the test is repeated with the addition of 0.1 ml of *arsenic standard solution (10 ppm As)*.

Stannous Chloride Solution, Dilute: Immediately before use, dilute 1 volume of *stannous chloride solution* with 10 volumes of *2 M hydrochloric acid*.

Starch: Of the Indian Pharmacopoeia.

Starch Iodide Solution; Starch-Iodide Solution: Dissolve 0.5 g of *soluble starch* in 100 ml with *water* containing 0.5 g of *potassium iodide*.

Starch, Soluble: Analytical reagent grade of commerce.

A 2 per cent w/v solution in hot *water* is at most slightly opalescent and remains fluid on cooling.

Starch Mucilage: Triturate 0.5 g of *starch* or *soluble starch* with 5 ml of *water* and add, stirring continuously, to sufficient *water* to produce about 100 ml. Boil for a few minutes, cool and filter.

Produces a blue colour with free iodine in the presence of a soluble iodide.

It must be freshly prepared.

Starch Solution: Triturate 1 g of *soluble starch* with 5 ml of *water* and add, stirring continuously, to 100 ml of boiling *water* containing 10 mg of *mercuric iodide*.

Complies with the following test which should be carried out each time the reagent is used.

SENSITIVITY TO IODINE — Mix 1 ml of the reagent with 20 ml of *water*, add about 50 mg of *potassium iodide* and 0.05 ml of *0.005 M iodine*; a blue colour is produced.

Starch Solution, Iodide-free: Triturate 1 g of *soluble starch* with 5 ml of *water* and add, stirring continuously, to 100 ml of boiling *water*.

Prepare immediately before use.

Staphylococcus Aureus Strain V8 Protease: Microbial extracellular proteolytic enzyme. A lyophilised powder containing 500 units to 1000 units per milligram of solid.

Starch Substrate: Stir an amount of *soluble starch* equivalent to 1.0 g of the dried substance in a beaker with 5 ml of *water*. Add, stirring continuously, to 75 ml of boiling *water*. Rinse the beaker with two quantities, each of 5 ml of *water*, add the washings to the hot starch solution and reboil for 2 minutes, stirring continously. Cool to 25°, dissolve 5 g of *sodium chloride* in the starch solution and add sufficient *water* to produce 100 ml. Dilute 10.0 ml of this solution to 100.0 ml with *phosphate buffer solution pH 6.0* in the analysis of bacterial amylase and with acetate *buffer pH 5.0* in the analysis of fungal amylase. 1 ml of starch substrate contains 1.0 mg of dry soluble starch.

It must be freshly prepared.

Stearic Acid: Of the Indian Pharmacopoeia.

Stearic Anhydride: $C_{36}H_{70}O_3 = 550.95$

General laboratory reagent grade of commerce.

White, waxy flakes; mp, about 70°.

Strontium Carbonate: $SrCO_3 = 147.6$

White or almost white, crystalline powder.

Strontium Chloride: $SrCl_2,6H_2O = 266.62$

Analytical reagent grade of commerce.

Colourless, odourless crystals or white granules; efflorescent in air; deliquescent in moist air.

Styrene-Divinylbenzene Cation-Exchange Resin: General chromatographic reagent grade of commerce suitable for use in column chromatography.

A strongly acidic, crossed-linked sulphonated resin containing about 2 per cent of *divinylbenzene*. White to light tan-coloured beads; relatively free flowing. Available in the hydrogen form in the 25 to 50, 45 to 100 and 80 to 270 mesh sizes or can be regenerated to the hydrogen form by treating with 5 per cent

v/v *hydrochloric acid*. Insoluble in *water*, in *methanol* and in *acetonitrile*.

Complies with the following tests.

MOISTURE CONTENT — Transfer 10 to 12 ml of the resin (as received) to a flask and convert it completely to the hydrogen form by stirring with 150 ml of a 5 per cent v/v solution of *hydrochloric acid* for not less than 30 minutes. Decant the acid and wash the resin in the same manner with *water* until the wash *water* is neutral to litmus.

Transfer 5 to 7 ml of the fully regenerated and expanded resin to a glass-filtering crucible and remove only the excess surface water by very careful suction. Transfer the conditioned resin to a tared weighing bottle and weigh. Dry in a vacuum oven at a pressure of about 5.5 kPa at 105°for 16 hours. Transfer from the vacuum oven to a desiccator, cool to room temperature and weigh again. The loss in weight is between 75 and 83 per cent.

TOTAL WET VOLUME CAPACITY — Transfer 3 to 5 ml of the regenerated and undried resin obtained in the test for MOISTURE CONTENT to a 5 ml graduated cylinder and fill it with *water*. Remove any air bubbles from the resin bed with a stainless steel wire and settle the resin to its minimum volume by tapping the graduated cylinder. Record the volume of the resin.

Transfer the resin to a 400 ml beaker, add about 5 g of *sodium chloride* and titrate, stirring well, with *0.1 M sodium hydroxide* to the blue end-point of *bromothymol blue*. Calculate the total wet volume capacity of the resin, which should be not less than 0.6 milliequivalents per ml, from the following expression.

$$\text{m.Eq.per ml} = \frac{\text{Net volume of NaOH(ml)} \times \text{Molarity}}{\text{ml of the resin}}$$

MESH SIZE — To 200 ml of *water* contained in a 1-litre wide-mouthed bottle or beaker add 150 ml of the resin under examination and allow to stand for at least 4 hours for complete swelling. Transfer 100 ml of the settled or swollen resin by means of a 100 ml graduated cylinder to the top screen of an appropriate set of brass sieves (see Appendix 2.1.3). Wash the resin on each sieve thoroughly with a jet of *water* until the resin is completely graded, collecting the wash *water* in a suitable container. Wash the beads of the resin remaining on each seive back into the 100 ml graduated cylinder and record the volume of resin settled on each sieve. Not less than 70 per cent of the resin is within the specified mesh size.

Styrene-Divinylbenzene Cation-Exchange Resin, Strongly Acidic: General chromatographic reagent grade of commerce (Dowex 50W-X8 is suitable).

Substrate Plasma Deficient in Clotting Factor V: Preferably use congenitally deficient plasma or, alternatively, prepare in the following manner. Separate the plasma from human blood collected in one-tenth of its volume of a 1.34 per cent w/v solution of *sodium oxalate* and incubate at 37° for 24 to 36 hours. This plasma should have a clotting time, when tested by the assay method given under *Clotting Factor V Solution*, of 70 to 100 seconds; if the clotting time is less than 70 seconds, incubate the plasma for a further 12 to 24 hours.

Store protected from light and moisture.

Sucrose: Of the Indian Pharmacopoeia.

Sulphaguanidine; Sulphguanidin,4-Amino-*N*-carbamimidoyl-benzenesulphonamide, (4-Amino-N-(aminoiminomethyl) benzenesulfonamide: $C_7H_{10}N_4O_2S = 214.24$

Analytical reagent grade of commerce.

Sulphamic Acid: $NH_2SO_3H = 97.09$

General laboratory reagent grade of commerce.

White crystals or crystalline powder; mp, about 205°, with decomposition.

4-Sulphamoylbenzoic Acid; *p*-Carboxybenzene sulphonamide; 4-Carboxybenzene Sulphonamide: $C_7H_7NO_4S = 201.20$

General laboratory reagent grade of commerce.

Flat, shiny prisms; mp, about 291°.

Sulphanilamide: $H_2NC_6H_4SO_2NH_2 = 172.21$

General laboratory reagent grade of commerce.

White or almost white, crystalline powder; mp, about 165°.

Store in a well-closed, light-resistant containers.

Sulphanilic Acid: $H_2NC_6H_4SO_3H = 173.19$

Sulphanilic Acid Solution. Dissolve 0.33 g of *sulphanilic acid* in 75 ml of *water* heating gently if necessary and dilute to 100 ml with *glacial acetic acid*.

Sulphanilic Acid Solution, Diazotised: Dissolve with warming, 0.9 g of *sulphanilic acid* in 9 ml of *hydrochloric acid* and dilute to 100 ml with *water*. Cool 10 ml of this solution in ice-bath and add 10 ml of an ice-cold 4.5 per cent w/v solution of *sodium nitrite*. Allow to stand in ice-bath for 15 minutes and immediately before use add 20 ml of *dilute sodium carbonate solution*.

Sulphathiazole: Of the Indian Pharmacopoeia.

Sulphomolybdic Acid Solution: Dissolve, with heating, 2.5 g of *ammonium molybdate* in 20 ml of *water*. Separately with care add 28 ml of *sulphuric acid* to 50 ml of *water* and cool. Mix the two solutions and dilute to 100 ml with *water*.

Store in polyethylene containers.

Sulphosalicylic acid; 2-Hydroxy-5-sulphobenzoic acid: $C_7H_6O_6S,2H_2O = 254.2$

A white crystalline powder or crystals, very soluble in *water* and in *ethanol*.

mp, about 109°.

Sulphur, Precipitated: Precipitated grade of commerce.

Pale, greyish yellow or greenish yellow, soft powder.

Sulphur Dioxide: SO_2 = 64.06

Laboratory cylinder grade of commerce.

Colourless gas; odour, acrid and penetrative.

Sulphuric Acid: H_2SO_4 = 98.07

Where no molarity is indicated, use analytical reagent grade of commerce containing about 98 per cent w/w of *sulphuric acid* and about 18M in strength.

Colourless, corrosive oily liquid; evolves much heat when added to *water*; wt. per ml, about 1.84 g.

Sulphuric Acid, x M: Solutions of any molarity x M may be prepared by carefully adding 54x ml of *sulphuric acid* to an equal volume of *water* and diluting to 1000 ml with *water*.

Sulphuric Acid, x per cent: Mix x ml of *sulphuric acid* carefully with *water*, cool and adjust the volume to 100 ml to produce the specified percentage v/v of *sulphuric acid*.

Sulphuric Acid, Dilute: Contains approximately 10 per cent w/w of H_2SO_4.

Dilute 57 ml of *sulphuric acid* to 1000 ml with *water*.

Sulphuric Acid, x M Ethanolic: Solutions of any molarity xM may be prepared by carefully mixing 54x ml of *sulphuric acid* with *ethanol (95 per cent)*, to produce 1000 ml.

Sulphuric Acid, x per cent Ethanolic: Mix x ml of *sulphuric acid* carefully with *ethanol (95 per cent)*, cool and adjust the volume to 100 ml to produce the specified percentage v/v of *ethanolic sulphuric acid*.

Sulphuric Acid-Formaldehyde Reagent: Mix 2 ml of *formaldehyde solution* with 100 ml of *sulphuric acid*.

Sulphuric Acid, x M Methanolic: Solutions of any molarity xM may be prepared by carefully mixing 54x ml *sulphuric acid* with *methanol* to produce 1000 ml.

Sulphuric Acid, x per cent Methanolic: Mix x ml *sulphuric acid* carefully with *methanol*, cool and adjust the volume to 100 ml to produce the specified percentage v/v of *methanolic sulphuric acid*.

Sulphuric Acid, Nitrogen-free: A grade of commerce containing not less than 96.0 per cent w/w of *sulphuric acid* and complying with the following test.

NITRATE — To 5 ml of *water* add carefully 45 ml of sample, cool to room temperature and add 8 mg of *N,N'-diphenylbenzidine*; the solution is colourless or very pale blue.

Tannic Acid; Tannin: $C_{76}H_{52}O_{46}$ = 1701.24

General laboratory reagent grade of commerce.

Yellowish white or light brownish glistening scales, masses or impalpable powder.

Store protected from light.

Tannic Acid Solution: A 10.0 per cent w/v solution of *tannic acid*. Prepare immediately before use.

Tartaric Acid: Of the Indian Pharmacopoeia.

Testosterone: $C_{19}H_{28}O_2$ = 288.41

General laboratory reagent grade of commerce.

mp, about 155°.

Testosterone Acetate: $C_{21}H_{30}O_3$ = 330.47

General laboratory reagent grade of commerce.

Testosterone Propionate: Of the Indian Pharmacopoeia

Tetrabutylammonium Bromide: $(C_4H_9)_4NBr$ = 322.38

General laboratory reagent grade of commerce.

White, crystalline powde; mp, about 104°.

Tetrabutylammonium Dihydrogen Orthophosphate; Tetrabutylammonium dihydrogen phosphate: $C_{16}H_{38}NO_4P$ = 339.5

General reagent grade of commerce.

mp, about 153°.

Store protected from moisture.

Tetrabutylammonium Hydrogen Sulphate: $C_{16}H_{37}NO_4S$=339.54

White, crystalline powder; mp, about 171°; contains not less than 97.0 per cent of $C_{16}H_{37}NO_4S$.

ASSAY — Dissolve about 0.170 g, accurately weighed, in 140 ml of *water* and titrate with *0.1 M sodium hydroxide*. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03395 g of $C_{16}H_{37}NO_4S$.

Complies with the following test.

ABSORBANCE (2.4.1) — Absorbance of a 5 per cent w/v solution in the range 240 nm to 300 nm, not more than 0.05.

Tetrabutylammonium Hydroxide: $C_{16}H_{37}NO,30H_2O$ = 800

General laboratory reagent grade of commerce.

Contains not less than 98.0 per cent w/v of $C_{16}H_{37}NO,30H_2O$.

ASSAY — Dissolve 1 g in 100 ml of *water* and titrate immediately with *0.1 M hydrochloric acid* determining the end point potentiometrically.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.08 g of $C_{16}H_{37}NO,30H_2O$.

Tetrabutylammonium Hydroxide Solution: General reagent grade of commerce containing 40.0 per cent w/v of $C_{16}H_{37}NO$.

Tetrabutylammonium Iodide: $[CH_3(CH_2)_3]_4NI = 369.37$

General laboratory reagent grade of commerce.

White or slightly coloured crystals or crystalline powder; mp, about 147°; contains not less than 98.0 per cent of $C_{16}H_{36}IN$.

Complies with the following test.

SULPHATED ASH (2.3.18) — Not more than 0.02 per cent.

ASSAY — Dissolve 1.2 g, accurately weighed, in 30 ml of *water* and add 50 ml to *0.1 M Silver Nitrate* and 5 ml of *2 M Nitric acid*. Titrate the excess of *silver nitrate* with *0.1 M ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.03694 g of $C_{16}H_{36}IN$.

1,1,2,2-Tetrachloroethylene; Tetrachloroethane: $C_2H_2Cl_4 = 167.84$

General laboratory reagent grade of commerce.

Heavy, mobile liquid; non-flammable; Odour, chloroform-like

1-Tetradecane; *n*-Tetradecane: $CH_3(CH_2)_{12}CH_3 = 198.39$

General laboratory reagent grade of commerce.

Clear, colourless liquid; refractive index at 20°, about 1.429; bp, about 253°; wt. per ml, about 0.76 g; contains not less than 99.5 per cent of $C_{14}H_{30}$.

Tetradecylammonium Bromide: $C_{40}H_{84}BrN = 659.0$.

Analytical reagent grade of commerce.

Melting point, 88° to 89°.

Tetrahydrofuran: $CH_2(CH_2)_2CH_2O = 72.11$

Analytical reagent grade of commerce.

Clear, colourless, flammable liquid; bp, about 66°; wt. per ml, about 0.888 g.

NOTE — Do not distil unless the tetrahydrofuran complies with the test for peroxides.

PEROXIDES — Place 8 ml of *starch iodide solution* in a 12 ml glass stoppered cylinder and about 1.5 cm in diameter. Fill completely with the reagent under examination, shake vigorously and allow to stand protected from light for 30 minutes; no colour is produced.

Tetrahydrofuran intended use for in spectrophotometry complies with the following additional reqirement.

TRANSMITTANCE — Not less than 20 per cent at about 255 nm, 80 per cent at about 270 nm and 98 per cent at about 310 nm determined using *water* as the blank.

Tetramethylammonium Chloride: $(CH_3)_4NCl = 109.60$

Colourless crystals; contains not less than 98.0 per cent of $C_4H_{12}NCl$

ASSAY — Transfer about 200 mg, acurately weighed, to a beaker, add 50 ml of *water* and 10 ml of *dilute nitric acid*, swirl to dissolve, add 50 ml of *0.1 M silver nitrate* and mix. Add 2 ml of *ferric ammonium sulphate solution* and 5 ml of *nitrobenzene*, shake and titrate the excess silver nitrate with *0.1 M ammonium thiocyanate*.

1 ml of *0.1 M silver nitrate* is equivalent to 0.01096 g of $(CH_3)_4NCl$.

Tetraheptylammonium Bromide: $C_{28}H_{60}BrN = 490.7$.

Analytical reagent grade of commerce.

Melting point, 89° to 91°.

Tetramethylammonium Hydrogen Sulphate: $C_4H_{13}NO_4S = 171.21$

Chromatographic grade of commerce.

Tetramethylammonium Hydrogen Sulphate intended for use in high performance liquid chromatography complies with the following test.

TRANSMITTANCE — Not less than 50 per cent at about 200 nm and 90 per cent at about 220 nm determined using a *0.005 M solution*.

Tetramethylammonium Hydroxide: $C_4H_{13}NO = 91.15$

Commercially available as approximately 25 per cent w/v aqueous solution or as the crystalline pentahydrate with strong ammonia-like odour. Tetramethylammonium hydroxide is a stronger base than ammonia and absorbs carbon dioxide from air rapidly.

Store protected from moisture.

ASSAY — Weigh accurately a glass-stoppered flask containing about 15 ml. of *water* and add solution of *tetramethylammonium hydroxide* equivalent to about 200 mg. of $(CH_3)_4NOH$ and weigh again. Add *methyl red* as indicator and titrate the solution with *0.1 M hydrochloric acid*.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.009115g of $C_4H_{13}NO$.

Tetramethylammonium Hydroxide Pentahydrate: $C_4H_{13}NO,5H_2O = 181.2$

General laboratory reagent grade of commerce.

mp, about 66°.

Tetramethylammonium Hydroxide Solution; Tetramethylammonium Hydroxide solution (10 per cent).

General laboratory reagent grade of commerce.

Clear, colourless or very pale liquid; odour; strongly ammoniacal; contains not less than 10.0 per cent w/w of $C_4H_{13}NO$.

ASSAY — To 1 g add 50 ml of *water* and titrate with *0.05 M sulphuric acid* using *methyl red solution* as indicator.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.009115 g of $C_4H_{13}NO$.

Tetramethylethyldiamine. $C_6H_{16}N_2 = 116.2$

A colourless liquid, miscible with *water* and *alcohol*.

wt. per ml. 0.78g; refractive index at 20°, 1.418; bp. 121°.

***N,N,N',N'*-Tetramethyl-*p*-phenylenediamine Dihydrochloride**; *N,N,N',N'*-Tetramethyl-*p*-phenylenediammonium Dichloride: $C_6H_4[N(CH_3)_2]_2,2HCl = 237.17$

General laboratory reagent grade of commerce.

White or slightly tinted, crystalline powder.

1,2,3,4-Tetraphenylcyclopenta-1,3-dienone; 1,2,3,4-D-Tetraphenyl-1,3-Cyclopentadienone: $C_{29}H_{22} = 370.49$.

General laboratory reagent grade of commerce.

Off white to grey powder, darkens in storage; mp, about 178°.

Tetrazolium bromide; 3-(4,5-Dimethylthizol-2-yl)-2,5-diphenyltetrazolihm bromide: $C_{18}H_{16}BrN_5S = 414.3$.

Thebaine; (5*R*,9*R*,13*S*)-4,5 Epoxy-3,6-dimethoxy-9'-methylmorphina-6, 8-diene:$C_{19}H_{21}NO_3 = 311.38$

General laboratory reagent grade of commerce.

White or pale yellow powder; mp, about 193°

Theophylline: Of the Indian Pharmacopoeia.

Thiamazole; Methimazole; 2-Mercapto-1-methylimidazole: $C_4H_6N_2S = 114.17$

General laboratory reagent grade of commerce.

mp, about 145°.

Thiazole orange: $C_{26}H_{24}N_2O_3S_2 = 476.61$

General laboratory reagent grade of commerce.

Red to orange powder.

2-(2-Thienyl)acetic Acid; 2-Thiopheneacetic acid: $C_6H_6O_2S = 142.18$

General Laboratory reagent grade of commerce.

mp, about 65°.

Thioacetamide: $CH_3CSNH_2 = 75.13$

General laboratory reagent grade of commerce.

White crystals or crystalline powder; mp, about 113°

Thioacetamide Reagent: Add 1 ml of a mixture of 15 ml of *1 M sodium hydroxide*, 5 ml of *water* and 20 ml of *glycerin (85 per cent)* to 0.2 ml of *thioacetamide solution*, heat in a water-bath for 20 seconds cool and use immediately.

Thioacetamide Solution: A 4 per cent w/v solution of *thioacetamide*.

Thioglycollic Acid; Mercaptoacetic Acid: $HSCH_2COOH = 92.12$

Analytical reagent grade of commerce.

Colourless or nearly colourless liquid; odour strong and unpleasant; wt. per ml about 1.33 g.

Thiomersal: Of the Indian Pharmacopoeia.

Thiourea: $NH_2CSNH_2 = 76.12$

Analytical reagent grade of commerce.

Colourless, crystalline powder; odour, faint but characteristic; mp, about 178°.

Thoron; 1-*o*-Arsonophenylazo-2-naphthol-3,6-disulphonic Acid Sodium Salt; Thoronal: $C_{16}H_{10}AsN_2Na_3O_{10}S_2$(approx)

General laboratory reagent grade of commerce.

***L*-Threonine**: $C_4H_9NO_3 = 119.1$

Chromatographic reagent grade of commerce.

A white, crystalline powder; $[\alpha]_D^{20}$, about –28 (2 per cent w/v in *water*).

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin layer chromatography*, protected from light, using *silica gel G* as the coating substance and a mixture of 25 volumes of *water* and 75 volumes of *phenol* as the mobile phase but allowing the solvent front to ascend 12 cm from the line of application. Apply to the plate 5 µl of a 0.2 per cent w/v solution of the reagent being examined and keep the plate in the vapours of the mobile phase for 12 hours before development. After removal of the plate, dry it, spray with a 0.1 per cent w/v solution of *ninhydrin* in *butan-1-ol* saturated with *water* and heat at 100° to 105° for 10 minutes. The chromatogram shows only one spot.

Thrombin: Dried human thrombin obtained from liquid plasma. It may be prepared by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength and temperature.

General laboratory reagent grade of commerce.

Yellowish white powder.

Store in sealed, sterile light-resistant containers under nitrogen, at a temperature below 25°.

Thrombokinase Extract; Thromboplastin Reagent: Extract 1.5 g of acetone-dried ox brain with 60 ml of *water* for 10 to 15 minutes at 50°, centrifuge for 2 minutes at 1500 rpm and

decant the supernatant liquid. This extract will retain its activity for several days when stored in a refrigerator. It may contain 0.03 per cent w/v of *o-cresol* as an antimicrobial preservative.

Thromboplastin: A preparation containing the membrane glycoprotein tissue factor and phospholipid, either purified from animal brain (usually rabbit) or human placenta or manufactured using recombinant DNA technology with added phospholipid. The preparation is formulated for routine use in the prothrombin time test and may contain calcium.

Thymine; 5-Methylpyrimidine-2,4(1*H*,3*H*)-dione; $C_5H_6N_2O_2$ = 126.1

Analytical reagent grade of commerce.

Thymol: Of the Indian Pharmacopoeia.

Tin; Granulated Tin; Sn = 118.69

Analytical reagent grade of commerce.

Silver-white sticks or granules.

Complies with the following test.

ARSENIC — 1 g complies with the limit test for arsenic (2.3.10). (10 ppm).

Titanium Trichloride; Titanous Chloride; Titanium(III) Chrloride: $TiCl_3$ = 154.24

General labraotry reagent grade of commernce.

Reddish violet crystals; mp, about 440°.

Titanium Trichloride Solution

General laboratory reagent grade of commerce containing about 15 w/v of $TiCl_3$ in *hydrochloric acid* (10 per cent w/v HCl). Store the solution in tightly-closed, glass-stoppered, light-resistant bottles.

Toluene; Methylbenzene: $C_6H_5CH_3$ = 92.14

Analytical reagent grade of commerce.

Clear, colourless liquid; odour, characteristic; bp, about 110°; wt. per ml, about 0.87 g.

Toluene, Anhydrous: Toluene which has been dried over anhydrous sodium sulphate.

Toluene-3,4-dithiol-Zinc Complex: $C_7H_6S_2Zn$ = 219.62

General laboratory reagent grade of commerce.

Toluene, Prepared: Toluene prepared by first shaking *toluene* with a small quantity of *water*, separating the excess *water* and distilling the *toluene*.

Toluene-o-sulphonamide; 2-Methylbenzene-sulphonamide;Toluene-2-sulphonamide: $C_7H_9NO_2S$ = 171.21

General laboratory reagent grade of commerce.

mp, about 156°.

Toluene-4-sulphonamide; Toluene-*p*-sulphonamide; 4- Methylbenzenesulphonamide: $C_7H_9NO_2S$ = 171.21

General laboratory reagent grade of commerce.

mp, about 136°.

Complies with the following test.

HOMOGENEITY — Carry out the test for Related substances described in the monograph for Tolbutamide applying to the plate 5 µl of a 0.015 per cent w/v solution in *acetone*. The chromatogram shows only one spot.

Toluene- *p*-sulphonic Acid; Toluenesulphonic acid; 4-methylbenzenesulphonic acid: $C_7H_8O_3S,H_2O$ = 190.2

General reagent grade of commerce.

Not less than 87.0 per cent of $C_7H_8O_3S$.

A white, crystalline powder or crystals.

Tosylarginine Methyl Ester Hydrochloride; Methyl *N*-tosy-L-argininate hydrochloride: $C_{14}H_{22}N_2O_4S,H_2O$ = 378.9

Analytical reagent grade of commerce suitable for the assay of trypsin.

A white, crystalline solid; mp, about 145°.

Tosylphenylanychloromethane; *N*-Tosy-L-phenyanyl-chloromethane; L-tosylaminophenethylchloromethyl ketone: $C_{17}H_{18}ClNO_3S$ = 351.9

Analytical reagent grade of commerce.

A white, crystalline solid; mp, about 105°.

Triacetin; Glycerol Triacetate: $CH_2(COOCH_3)CH(COOCH_3)CH_2(COOCH_3)$ = 218.20

Clear, colourless or pale straw-coloured liquid; wt. per ml, about 1.156 g; contains not less than 95.0 per cent and not more than 99.0 per cent w/w of $C_9H_{14}O_6$.

ASSAY — Carry out the method for the determination of esters (2.3.25).

1 ml of *0.5 M ethanolic potassium hydroxide* is equivalent to 0.03637 g of $C_9H_{14}O_6$.

Triazolam; Chlorazam; 8-Chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4] benzodiazepine: $C_{17}H_{12}Cl_2N_4$ = 343.22

General laboratory reagent grade of commerce.

Tan Crystals; mp, about 160°.

Tributylphosphine: $C_{12}H_{27}P$ = 202.3

Clear, colourless liquid.

bp, about 240°.

mp, about -60°.

Tributyl Phosphate; Tributyl Orthophosphate: $[CH_3(CH_2)_3]_3PO_4$=266.32

General laboratory reagent grade of commerce.

Clear, colourless liquid; refractive index at 20°, about 1.425; wt.per ml, about 0.977 g.

It should be washed 3 times before use with one-sixth of its volume of a solution containing 10 per cent w/v *sodium chloride* and 1.3 per cent w/v of *sodium dihydrogen phosphate*.

Tricine; N-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine: $C_6H_{13}NO_5$=179.2.

Use electrophoresis-grade reagent; mp, about 183°.

Trichloroacetic Acid: Cl_3COOH=163.40

Analytical reagent grade of commerce.

Colourless, very deliquescent crystals or crystalline masses; odour, slight or pungent and characteristic; mp, about 56°.

Store protected from light.

Trichloroacetic Acid Solution: Dissolve 10 g of *trichloroacetic acid* in sufficient *water* to produce 100 ml.

1,1,1-Trichloroethane; Methyl Chloroform: CH_3CCl_3=133.42

Analytical reagent grade of commerce.

Colourless, heavy liquid; bp, about 74°; wt. per ml, about 1.32 g.

Tricosane: $C_{23}H_{48}$=324.6

General laboratory reagent grade of commerce.

White crystals, practically insoluble in *water*, soluble in *hexane*; mp, about, 48°.

Triethanolamine: $N(CH_2CH_2OH)_3$=149.18

General laboratory reagent grade of commerce.

Viscous, very hygroscopic, colourless liquid; odour, slightly ammoniacal; becomes brown on exposure to air and light; refractive index at 20°, about 1.484; wt. per ml, about 1.13 g.

Store protected from light.

Triethylamine; *N,N*-Diethylethanamine: $(C_2H_5)_3N$=101.19

General laboratory reagent grade of commerce.

Colourless liquid; odour, strongly ammoniacal; bp, about 90°; refractive index at 20°, about 1.401; wt.per ml, about 0.73 g.

Store protected from moisture.

Triethylamine Hydrochloride: $C_6H_{15}N,HCl$=137.65

General laboratory reagent grade of commerce.

White to off white powder.

mp, about 256° with decomposition.

Triethylenediamine; 1,4-Diazobicyclo[2.2.2]octane: $C_6H_{12}N_2$ =112.17

General laboratory reagent grade of commerce.

Hygroscopic crystals; sublimes readily at room temperature; mp, about 158°; bp, about 174°

Store in tightly-closed containers.

Trifluoroacetic acid: $C_2HF_3O_2$=114.0

Content. Minium 99 per cent of $C_2HF_3O_2$.

Liquid, miscible with *acetone* and with *alcohol*; wt. per ml, about 1.53g; bp, about 72°.

Use a grade suitable for protein sequencing.

Store protected from moisture.

Trifluoroacetic Anhydride: $C_4F_6O_3$=210.0

General laboratory reagent grade of commerce.

A colourless liquid; d_{20}^{20}, about 1.5.

Trimethylchlorosilane; Chlorotrimethylsilane: $(CH_3)_3SiCl$ = 108.65

General laboratory reagent grade of commerce.

Clear, colourless liquid; bp, about 57°; refractive index at 20°, about 1.460; wt. per ml, about 0.86 g

2,2,4-Trimethylpentane; Iso-octane$(CH_3)_2CHCH_2C(CH_3)_3$ = 114,23

General laboratory reagent grade of commerce.

Colourless, liquid; flammable; bp, about 99°; refractive index at 20°, about 1.392; wt. per ml, about 0.694 g.

2,2,4-Trimethylpentane intended for use in spectrophotometry complies with following additional test.

TRANSMITTANCE — Not less than 98 per cent between 250 and 420 nm using *water* as the blank.

2,4,6-Trinitrobenzenesulphonic Acid; Picrylsulphonic acid: $C_6H_3N_3O_9S,3H_2O$=347.2

General reagent grade of commerce.

mp, 190° to 195°.

Triphenylamine: $C_{18}H_{15}N$=245.32

General laboratory reagent grade of commerce.

White, crystalline solid; mp, about 126°.

Tris Buffered Saline (TBS). Dissolve 2.4 g of *tris*, 8 g of *sodium chloride* in 100 ml of *water* and adjusted the pH to 7.6 with *hydrochloric acid*.

TBST. *Tris buffered saline (TBS)* containing 0.1 per cent of *tween 80*.

Tris(hydroxymethyl)aminomethane; Tromethamine; Trometamol; THAM; *Tris* (hydroxymethyl) methylamine; 2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3 = 121.13$

Analytical reagent grade of commerce.

White crystals or crystalline powder; odour, characteristic; mp, about 170°.

Tris (hydroxymethyl) aminomethane reagent: Weigh accurately 1.5g of *tris (hydroxymethyl) aminomethane* in 100 ml volumetric flask, dissolve and dilute to volume with *water*, and mix. Transfer 40 ml of this solution to a 200 ml volumetric flask and dilute to volume with *dimethyl sulphoxide*. Use this reagent within 4 hours.

Tropic Acid; (2*RS*)-3-hydroxy-2-phenylpropanoic acid: $C_9H_{10}O_3 = 166.2$

Trypsin: Freeze-dried, salt-free beef trypsin containing not less than 9000 benzoylarginine ethyl ester units per mg.

Proteolytic enzyme obtained by activation of tyrpsinogen extracted from the pancreas of beef (Bos taurus Linn).

Store at a temperature not exceeding 4°.

Trypsin for Peptide Mapping:

Trypsin of high purity treated to eliminate chymotryptic activity.

Tyramine; 4-(2-Aminoethyl)phenol: $C_8H_{11}NO = 137.18$.

General laboratory reagent grade of commernce.

mp, about 162°.

L-Tyrosine; Tyrosine: $OHC_6H_4CH_2CH(NH_2)COOH = 181.19$

General laboratory reagent grade of commerce.

White powder.

Complies with the following test.

HOMOGENEITY — Carry our the test for Related substances described in the monograph of Levodopa. The chromatogram shows only one spot.

Undecenoic Acid: Of the Indian Pharmacopoeia.

Uranyl Acetate: $U_2(CH_3CO)_2,2H_2O = 424.15$

General laboratory reagent grade of commerce.

Bright yellow, crystalline powder; odour, slightly acidic.

Urea: Of the Indian Pharmacopoeia.

Urease-active Meal: General laboratory reagent grade of commerce. Complies with the following test.

ACTIVITY — 1 mg hydrolyses 3 mg of urea in 30 minutes at 37°

Uridine; 1-β-D-Ribofuranosyluracil; Uracil Riboside: $C_9H_{12}N_2O_6 = 244.20$

Chromatographic grade of commerce.

mp, about 165°.

Valeric Acid; *n*-Valeric Acid; Pentanoic Acid: $CH_3(CH_2)_3COOH = 102.13$

General laboratory reagent grade of commerce.

Colourless liquid; odour, unpleasant; refractive index at 20°, about 1.409; bp, about 186°; wt. per ml, about 0.94 g.

Vanillin: Of the Indian Pharmacopoeia.

Vanillin glacial acetic acid reagent: Equal volumes of 5 per cent w/v solution of Vanillin in *glacial acetic acid* and 10 per cent w/v solution of *sulphuric acid* in *water*. Use freshly prepared reagent.

Vanillin Solution, Phosphoric: Dissolve 1 g of *vanillin* in 25 ml of *ethanol (95 per cent)* and add 25 ml of *water* and 35 ml of *orthophosphoric acid*.

Vanillin Sulphuric Acid: Mix 98 volumes of a 1.0 per cent w/v solution of vanillin in *ethanol (95 per cent)* and 2 volumes of *sulphuric acid*.

Vanillin Sulphuric Acid Solution: A 5.0 per cent w/v solution of *vanillin* in *sulphuric acid*.

NOTE—Vanillin sulphuric acid solution should be freshly prepared.

Veratric Acid; 3,4-Dimethoxybenzoic Acid; $C_9H_{10}O_4 = 182.17$

General laboratory reagent grade of commerce.

mp, about 180°.

2-Vinylpyridine: $C_7H_7N = 105.1$

Yellow liquid, miscible in *water*.

d_{20}^{20}, about 0.97; n_D^{20}, about 1.549.

4-Vinylpyridine; 4-Ethenylpyridine: $C_7H_7N = 105.1$

Clear, deep yellowish-brown liquid; mp, 58° to 61°.

Wash Solution pH 2.5: To 500 ml of a 1 per cent v/v solution of *nitric acid* add *strong ammonia solution* until pH of the solution is 2.5. To the resulting solution add 10 ml of *buffer sollution pH 2.5* and mix.

Water: Purified water of the Indian Pharmacopoeia.

Water, Ammonia-free: To 100 ml of *water* add 0.1 ml of *sulphuric acid* and distil discarding the first 10 ml and collecting the following 50 ml.

Complies with the following test.

To 50 ml, add 2 ml of *alkaline potassium mercuri-iodide solution*; no colour is produced.

Water, Carbon Dioxide-free: Water which has been boiled vigorously for a few minutes and protected from the atmosphere during cooling and storage.

Water, Distilled: Purified Water of the Indian Pharmacopoeia that has been prepared by distillation.

Water for Injection: Of the Indian Pharmacopoeia.

Water for Injection, Sterile: Of the Indian Pharmacopoeia.

Water, Nitrate free: To 100 ml of *water* add about 5 mg each of *potassium permanganate* and *barium hydroxide* and distil discarding the first 10 ml and collecting the following 50 ml.

Water Purified: Of the Indian Pharmacopoeia

Wool Fat: Of the Indian Pharmacopoeia

Xanthydrol; 9-Hydroxyxanthene; Xanthen-9-ol; $C_{13}H_{10}O_2$ = 198.22

General laboratory reagent grade of commerce.

White to pale yellow powder; mp, about 123°; contains not less than 90.0 per cent of $C_{13}H_{10}O_2$.

Xanthydrol is also available as a solution in *methanol* containing 9.0 to 11.0 per cent w/v $C_{13}H_{10}O_2$

ASSAY — In a 250 ml flask dissolve 0.3 g in 3 ml of *methanol* or use 3 ml of solution. Add 50 ml of *glacial acetic acid* and add, dropwise with shaking, 25 ml of a 2 per cent w/v solution of *urea*. Allow to stand for 12 hours, collect the precipitate on a sintered glass filter (16 mm), wash with 20 ml of *ethanol (95 per cent)*, dry the precipitate at 105° and weigh.

1 g of the precipitate is equivalent to 0.9429 g of the $C_{13}H_{10}O_2$.

Store protected from light. If a methanolic solution is used, store in small, sealed ampoules and filter before use, if necessary.

Xanthydrol Reagent: Dissolve about 0.125 g of *xanthydrol* in 100 ml of *anhydrous glacial acetic acid*. Add 1 ml of *hydrochloric acid* immediately before use.

Xylene; Mixture of o-, m- and p-isomers; $C_6H_4(CH_3)_2$ = 106.17

Analytical reagent grade of commerce.

Colourless, clear, flammable liquid; bp, about 140°; wt. per ml, about 0.855 g.

***m*-Xylene**; 1,3-Dimethylbenzene: C_8H_{10} = 106.2

General laboratory reagent grade of commerce.

d_{20}^{20}, about 0.884; n_D^{20}, about 1.497; bp, about 139°; mp, about −47°.

Xylose: Of the Indian Pharmacopoeia.

Zinc: Zn = 65.38

Analytical reagent grade of commerce.

Silver-white cylinders, granules, pellets or filings with a blue sheen; contains not less than 99.5 per cent of Zn.

Complies with the following test.

ARSENIC — To 5 g add 15 ml of *hydrochloric acid* and 25 ml of *water*. Add 0.1 ml of *stannous chloride solution AsT* and 5 ml of *0.1 M potassium iodide*. Carry out the limit test for arsenic (2.3.10); no stain is produced on the mercuric chloride paper.

ACTIVITY — Repeat the test for ARSENIC using the same reagents and adding 1 ml of *arsenic standard solution (1 ppm As)*; a distinct stain appears on the *mercuric chloride paper*.

Zinc Acetate: $(C_2H_3O_2)_2Zn,2H_2O$ = 219.5

General reagent grade of commerce.

mp, about 237°.

Zinc Acetate, 0.25 M: Mix 600 ml of *water* with 150 ml of *glacial acetic acid* and 54.9 g of *zinc acetate*, stir to dissolve. While stirring, add 150 ml of *ammonium hydroxide*, cool to room temperature, and adjust to pH of 6.4 with *ammonium hydroxide* and dilute to 1000 ml with *water*.

Zinc, Activated: Cover a quantity of *zinc* with a solution containing 50 µg of *chloroplatinic acid* per ml. Allow to stand for 10 minutes, wash, drain and dry immediately.

Complies with the following tests.

ARSENIC — To 5 g add 15 ml of *hydrochloric acid* and 25 ml of *water*. Add 0.1 ml of *stannous chloride solution AsT* and 5 ml of *0.1 M potassium iodide*. Carry out the limit test for arsenic (2.3.10); no stain is produced on the mercuric chloride paper.

ACTIVITY — Repeat the test for ARSENIC using the same reagents and adding 1 ml of *arsenic standard solution (1 ppm As)*; a distinct stain appears on the *mercuric chloride paper*.

Zinc AsT: Granulated zinc which complies with the following additional test. To 10 g add 15 ml of *stannous chloride solution AsT* and 5 ml of *0.1 M potassium iodide*. Use the apparatus and method described in Appendix 2.3.10, but continue the action for 1 hour; no visible stain is produced on *mercuric chloride paper*. Repeat the test with the addition of 0.1 ml of *arsenic standard solution (10 ppm)*; a faint but distinct stain is produced.

Zinc Bis(diphenyldithiocarbamate): $C_6H_{12}N_2S_4Zn$ = 305.8

General laboratory reagent grade of commerce.

White odourless powder, mp, about 250°.

Zinc and Sodium Carbonate Reagent: Mix 1 part of *anhydrous sodium carbonate* and 2 parts of *zinc powder* and moisten the mixture with *anhydrous methanol*. Dry the mixture first on a water-bath and then at about 115° for a few hours.

Store in tightly-closed containers.

Zinc Chloride: Of the Indian Pharmacopoeia.

Zinc Chloride-Formic Acid solution; Zinc Chloride Solution: Dissolve 20 g of *zinc chloride* in 80 g of an 85 per cent w/v solution of *anhydrous formic acid*.

Zinc Chloride Solution, Iodinated: Dissolve 20 g of *zinc chloride* and 6.5 g of *potassium iodide* in 10.5 ml of *water*.

Add 0.5 g of iodine and shake for 15 minutes, filter if necessary.

Store protected from light.

Zinc Dithiol Reagent: Dissolve 0.2 g of *toluene-3,4-dithiol-zinc complex* in a 1 per cent w/v solution of *sodium hydroxide* containing 0.25 ml of *ethanol (95 per cent)*. Add 1 ml of *thioglycollic acid* and sufficient of the sodium hydroxide solution to produce 100 ml.

Prepare immediately before use.

Zinc, Granulated: Zn = 65.38

Bright silver-grey,metallic granules.

Zinc Powder; Zinc dust: Zn = 65.38

Analytical reagent grade of commerce.

Dense bluish grey powder; contains not less than 95.0 per cent of Zn.

Zinc Shot: Zn = 65.38

Analytical reagent grade of commerce.

Shot, 0.5 mm to 2.0 mm (about 8 to 30 mesh).

Zinc Sulphate: Of the Indian Pharmacopoeia.

Zinc Sulphate, x M: Solutions of any molarity x M may be prepared by dissolving 289x g of *zinc sulphate* in sufficient *water* to produce 1000 ml.

Zinc Sulphate Solution: A 0.011 per cent w/v solution of *zinc sulphate*; contains 0.025 mg of Zn in 1 ml.

Zincon; 1-(2-Hydroxy-5-sulphophenyl)-3-phenyl-5-(2-carboxyphenyl)formazan: $C_{20}H_{16}N_4O_6S$ = 440.43.

Dark purple powder; mp, about 215°.

Complies with the following tests.

SULPHATED ASH (2.3.18) — Not more than 2.0 per cent.

SENSITIVITY TO ZINC — Add 0.2 ml of a 0.08 per cent w/v solution in *alkaline borate buffer pH 9.0* and 10 ml of *alkaline borate buffer pH 9.0* to 10 ml of a solution containing 0.0001 per cent zinc contained in a Nessler cylinder; a pale blue colour is observed when compared with a solution containing 10 ml of *water* in place of the zinc solution.

Zincon Solution: Dissolve 0.130 g of *zincon* in 2 ml of *1 M sodium hydroxide* and dilute to 100 ml with *water*.

Zinc Undecenoate: Of the Indian Pharmacopoeia.

Zirconyl Nitrate: Approximately $ZrO(NO_3)_2$ = 231.23

General laboratory reagent grade of commerce.

Usually contains about 44.5 per cent of ZrO_2.

Zirconyl Nitrate Solution: Dissolve 0.1 g of Zirconyl Nitrate in a mixture of 60 ml of hydrochloric acid and 40 ml of *water*.

4.3. Indicators and Indicator Test Papers

A. Indicators

In the test and assays of the Pharmacopoeia, indicators are required to indicate the completion of a chemical reaction in volumetric analysis or to indicate the pH of solutions. Indicators may be substituted for one another provided the colours change over approximately the same range of pH but in the event of doubt or dispute as to the equivalence of indicators for a particular procedure, the indicator specified in the individual monograph is alone authoritative.

Any solvent required in a determination or test in which an indicator is specified should be previously neutralized to the indicator unless a blank determination is performed or specified.

Given below are materials which are to be used as indicators and the manner in which solutions of indicators are to be prepared.

Table 1 lists the more commonly used pH indicators in ascending order of the lower limit of their range with the corresponding colour changes.

Alizarin Red S; CI 58005; Alizarin S; Mordant Red B; Sodium Alizarine Sulphonate; 9,10-dihydro-3,4-dihydroxy-9,10-dioxo-2-anthrancesulphonic Acid Monosodium salt: $C_{14}H_7NaO_7S,H_2O$ = 360.27

General laboratory reagent grade of commerce.

Yellowish brown or orange-yellow powder.

Alizarin Red S Solution; Alizarin S Solution: A 0.1 per cent w/v solution of *alizarin red S*. Complies with the following test.

SENSITIVITY TO BARIUM — To 5 ml of *0.05 M sulphuric acid* add 5 ml of *water*, 50 ml of *acetate buffer pH 3.7* and 0.5 ml of the solution under examination. Add, dropwise, *0.05 M barium perchlorate*; the colour changes from yellow to orange-red.

Azo Violet; Magneson; 4-(*p*-Nitrophenylazo)resorcinol: $C_{12}H_9N_3O_4$ = 259.22

Red powder; mp, about 193°, with decomposition.

Brilliant Green; CI 42040; Malachite Green G; Basic Green 1: $C_{27}H_{34}N_2O_4S$ = 482.64

Technical Grade of commerce.

Small, glistening golden crystals.

Brilliant Green Solution: A 0.5 per cent w/v solution of *brilliant green* in *glacial acetic acid.*

Bromocresol Green; Bromocresol Blue; 4,4-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromo-*m*-cresol) *S,S*-dioxide: $C_{21}H_{14}Br_4O_5S$ = 698.01

White or pale buff-coloured powder.

TABLE 1-pH ranges and colour changes of indicators

Indicator	pH range	Colour change
Cresol Red	0.2 to 1.8 and 7.2 to 8.8	Red to Yellow Yellow to Red
Metacresol Purple	0.5 to 2.5 and 7.5 to 9.2	Red to Yellow Yellow to Violet
Thymol Blue	1.2 to 2.8 and 8.0 to 9.6	Red to yellow Yellow to Violet-blue
Metanil Yellow	1.2 to 2.3	Magenta to Yellow
Quinaldine Red	1.4 to 3.2	Colourless to Red
Dimethyl Yellow	2.8 to 4.6	Red to Yellow
Bromophenol Blue	2.8 to 4.6	Yellow to Blue-violet
Methyl Orange	2.9 to 4.0	Red to Yellow
Congo Red	3.0 to 5.0	Blue to Red
Bromocresol Green	3.6 to 5.2	Yellow to Blue
Methyl Red	4.2 to 6.3	Red to Yellow
Litmus	5.0 to 8.0	Red to Blue
Bromocresol Purple	5.2 to 6.8	Yellow to Blue-violet
Bromothymol Blue	6.0 to 7.6	Yellow to Blue
Neutral Red	6.8 to 8.0	Red to Orange
Phenol Red	6.8 to 8.4	Yellow to Red
Phenolphthalein	8.3 to 10.0	Colourless to Red
Thymolphthalein	9.3 to 10.5	Colourless to Blue
Titan Yellow	12.0 to 13.0	Yellow to Red

Bromocresol Green Reagent: Prepare a phosphate buffer solution by dissolving 43.0 g of *sodium dihydrogen phosphate* and 2.0 g of *anhydrous sodium phosphate* in sufficient *water* to make 1000 ml. Dissolve 0.2 g of *bromocresol green* in 30 ml of *water* and 6.5 ml of *0.1 M sodium hydroxide*. Dilute to 500 ml with the phosphate buffer solution, mix and adjust the pH to 4.6 with *0.1 M hydrochloric acid.*

Bromocresol Green Solution: Dissolve 50 mg of *bromocresol green* in 0.72 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.2 ml of the solution and 100 ml of *carbon dioxide-free water* is blue. Not more than 0.2 ml of *0.02 M hydrochloric acid* is required to change the colour of the solution to yellow.

Bromocresol Green-Methyl Red Solution. Dissolve 0.15 g of *bromocresol green* and 0.1 g of *methyl red* in 180 ml of *absolute ethanol* and dilute to 200 ml with *water.*

Bromocresol Purple; 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene) bis(6-bromo-*o*-cresol) *S,S*-dioxide: $C_{21}H_{16}Br_2O_5S$ = 540.23

White to pink, crystalline powder.

Bromocresol Purple Solution: Dissolve 50 mg of *bromocresol purple* in 0.92 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.2 ml of the solution and 100 ml of *carbon dioxide-free water* to which 0.05 ml of *0.02 M sodium hydroxide* has been added is bluish violet. Not more than 0.2 ml of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Bromocresol Purple Solution, Phosphate-buffered: Dissolve 43 g of *potassium dihydrogen phosphate* and 5 g of *sodium phosphate* in *water* to make 1000 ml. Adjust the pH of the solution to 5.3 ± 0.1, if necessary (Solution A). Dissolve 0.4 g of *bromocresol purple* in 30 ml of *water*, add 6.3 ml of *0.1 M sodium hydroxide* and dilute with *water* to make 500 ml (Solution B). Shake equal volumes of solution A, solution B and *chloroform* in a separator, allow the layers to separate and discard the chloroform. If appreciable colour is extracted by the chloroform, repeat with additional quantities of *chloroform* until no further colour is extracted.

Bromophenol Blue; 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)-bis(2,6-dibromophenol) *S,S*-dioxide: $C_{19}H_{10}Br_4O_5S$ = 669.96

Pinkish crystals.

Bromophenol Blue Solution; Strong Bromophenol Blue Solution; Ethanolic Bromophenol Blue Solution: Dissolve 0.1 g of *bromophenol blue* with gentle heating in 1.5 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)* and add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.05 ml of the solution and 20 ml of *carbon dioxide-free water* to which 0.05 ml of *0.1 M hydrochloric acid* has been added is yellow. Not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour to bluish violet.

Bromophenol Blue Reagent: Dissolve 50 mg of *bromophenol blue* with gentle heating in 3.73 ml of *0.02 M sodium hydroxide* and dilute to 100 ml with *water*.

Bromothymol Blue; 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)-bis(2-bromothymol) *S,S*-dioxide: $C_{27}H_{28}Br_2O_5S = 624.39$.

Cream-coloured powder.

Bromothymol Blue Solution; Aqueous Bromothymol Blue Solution: Dissolve 50 mg of *bromothymol blue* in 4 ml of *0.02 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.3 ml of the solution and 100 ml of *carbon dioxide-free water* is yellow. Not more than 0.1 ml of *0.02 M sodium hydroxide* is required to change the colour to blue.

BRP Indicator Solution: Dissolve 0.1g of *bromothymol blue*, 20 mg of *methyl red* and 0.2 g of *phenolphthalein* in sufficient *ethanol (95 per cent)* to produce 100 ml and filter.

Calcon; CI 15705; Solochrome Dark Blue; Mordant Black 17; Sodium 2-hydroxy-1-(2-hydroxy-1-naphthylazo)-naphthalene-4-sulphonate: $C_{20}H_{13}N_2NaO_5S = 416.38$

General laboratory reagent grade of commerce.

Brownish black powder with a violet sheen. Gives a purple-red colour with calcium ions in alkaline solution. When metal ions are absent, for example, in the presence of an excess of disodium edetate, the solution is blue.

Calcon Mixture: A mixture of 1 part of *calcon* with 99 parts of freshly ignited *anhydrous sodium sulphate*.

Complies with the following test.

SENSITIVITY — Dissolve 0.1 g in 2.5 ml of *water*. To 1 ml of the solution add 50 ml of *water*, 10 ml of *1 M sodium hydroxide* and 1 ml of a 1 per cent w/v solution of *magnesium sulphate*; the solution is blue. Add 0.1 ml of a 0.15 per cent w/v solution of *calcium chloride*; the solution becomes violet and on subsequent addition of 0.1 ml of *0.01 M disodium edetate* turns to blue again.

Calconcarboxylic Acid. Patton and Reeder's reagent; 2-hydroxy-1-(2-hydroxy-4-sulpho-1- naphthylazo)naphthalene-3-carboxylic acid; $C_{21}H_{14}N_2O_7S,3H_2O = 492.5$

General laboratory reagent grade of commerce.

A brownish black powder. Produces a sharp colour change from red to blue in titrations of calcium with disodium edetate.

Calconcarboxylic Acid Triturate. A mixture of 1 part of *calconcarboxylic acid* and 99 parts of *sodium chloride*.

Complies with the following test.

SENSITIVITY TO CALCIUM — Dissolve 50 mg in a mixture of 100 ml of *water* and 2 ml of *10M sodium hydroxide*; a blue colour is produced. Add 1 ml of a 1 per cent w/v solution of *magnesium sulphate* and 0.1 ml of a 0.15 per cent w/v solution of *calcium chloride*; a violet colour is produced. Add 0.15 ml of *0.01M disodium edetate*; a pure blue colour is produced.

Congo Red; CI 22120; Disodium (4,4'-biphenylbis-2,2-azo) bis(1-aminonaphthalene-4-sulphonate): $C_{32}H_{22}N_6Na_2O_6S_2 = 696.66$

Dark red or reddish brown powder. Decomposes on exposure to acid fumes.

Soluble in *water*.

Congo Red Fibrin: Soak washed and shredded *fibrin* overnight in a 2 per cent w/v solution of *congo red* in *ethanol (90 per cent)*, strain, wash the product with *water* and store under *ether*.

Congo Red Paper: Immerse strips of filter paper for a few minutes in *congo red solution*. Allow to dry.

Congo Red Solution: Dissolve 0.1 g of *congo red* in a mixture of 20 ml of *ethanol (95 per cent)* and *water* and dilute to 100 ml with *water*.

Complies with the following test.

SENSITIVITY — To 0.2 ml of the *congo red solution* add 100 ml of *carbon dioxide-free water* and 0.3 ml of *0.1M hydrochloric acid*. The solution is blue. Not more than 0.3 ml of *0.1M sodium hydroxide* is required to change the colour to pink.

Cresol Red; 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene) di-*o*-cresol *S,S*-dioxide: $C_{21}H_{18}O_5S = 382.44$

Red brown powder.

Cresol Red Solution: Warm 0.1 g of *cresol red* in a mixture of 2.65 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* to which 0.15 ml of *0.02 M sodium hydroxide* has been added is purplish red. Not more than 0.15 ml of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Crystal Violet; CI 42555; Basic Violet 3; Hexamethyl-*p*-rosaniline Chloride: $C_{25}H_{30}ClN_3 = 407.98$

When used for titrations in non-aqueous media, changes from violet (basic) through blue-green (neutral) to yellowish green (acidic).

Crystal Violet Solution: A 0.5 per cent w/v solution of *crystal violet* in *anhydrous glacial acetic acid*.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 50 ml of *anhydrous glacial acetic acid* is bluish purple. Add 0.1 ml of *0.1 M perchloric acid*; the solution turns blue-green.

Dimethyl Yellow; CI 11020; 4-Dimethylaminoazobenzene:

$C_{14}H_{15}N_3 = 225.29$

Yellow crystalline leaflets; mp, about 116°.

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin-layer chromatorgraphy* (2.4.17), using *silica gel G* as the coating susbtance and *dichloromethane* as the mobile phase. Apply to the plate 10 µl of a 0.01 per cent w/v solution in *dichloromethane*. The chromatogram shows only one spot.

Dimethyl Yellow Solution: A 0.2 per cent w/v solution of *dimethyl yellow* in *ethanol (90 per cent)*.

Complies with the following test.

SENSITIVITY — A solution containing 2 g of *ammonium chloride* in 25 ml of *carbon dioxide-free water*, to which is added 0.1 ml of the *dimethyl yellow solution*, is yellow. Not more than 0.1 ml of *0.1 M hydrochloric acid* is required to change the colour to red.

Dimethyl Yellow-Oracet Blue B Solution; Dimethyl Yellow-Solvent Blue 19 Solution: Dissolve 15 mg of *dimethyl yellow* and 15 mg of *oracet blue B* in *chloroform* and dilute to 500 ml with *chloroform*.

Eosin; CI 45380; Acid red 87: $C_{20}H_6Br_4Na_2O_5 = 691.86$

General laboratory reagent grade of commerce.

Red powder.

Eosin Solution: A 0.5 per cent w/v solution of *eosin* in *water*.

Eriochrome Black T; CI 14645; Mordant Black II; Solochrome Black; Sodium 1-(1-hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sulphonate: $C_{20}H_{12}N_3NaO_7S = 461.38$

General laboratory reagent grade of commerce.

Brownish black powder having a faint, metallic sheen. Gives a red colour with calcium, magnesium, zinc and certain other metals in alkaline solutions. When metal ions are absent, for example in the presence of an excess of disodium edetate, the solution is blue.

Store protected from light and moisture.

Complies with the following test.

SENSITIVITY — To 10 ml of a 1 in 200,000 solution in a mixture of equal parts of *methanol* and *water* add a 1 per cent w/v solution of *sodium hydroxide* until the pH is 10; the solution is pure blue in colour and free from cloudiness. Add 0.01 ml of *0.05 M magnesium sulphate*; the colour of the solution changes to red-violet, and on adding more *0.05 M magnesium sulphate*, the solution becomes wine-red in colour.

Eriochrome Black T Mixture; Eriochrome Black T Triturate; Mordant Black II Mixture: A mixture of 1 part of *eriochrome black T* and 99 parts of *sodium chloride*.

Store protected from light and moisture.

Complies with the following test.

SENSITIVITY — Dissolve 50 mg in 100 ml of *water*, a brownish violet colour is produced. Add 0.3 ml of *6 M ammonia;* the colour changes to blue. Add 0.1 ml of a 1 per cent w/v solution of *magnesium sulphate*; the colour changes to violet.

Eriochrome Black T Solution; Mordant Black II Soluton: Dissolve 0.2 g of *eriochrome black T* and 2 g of *hydroxylamine hydrochloride* in sufficient *methanol* to produce 50 ml.

Prepare immediately before use.

Fast Blue B Salt; CI 37235: $C_{14}H_{12}Cl_2N_4O_2 = 339.18$

General laboratory reagent grade of commerce.

Dark green powder, stabilised by the addition of zinc chloride.

Store protected from moisture in a cold place.

Ferroin Solution; Ferroin Sulphate Solution; Tris-(1,10-phenanthroline)ferrous Sulphate Complex: Dissolve 0.7 g of *ferrous sulphate* and 1.5 g of *1,10-phenanthroline hydrochloride* in 70 ml of *water* and add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — Add 0.1 ml of the solution and 0.15 ml of *osmic acid solution* to 50 ml of *1 M sulphuric acid*. Add 0.1 ml of *0.1 M ceric ammonium nitrate*; the colour changes from red to light blue.

Hydroxy Naphthol Blue; (1-(2-Naphtholazo-3,6-disulphonic Acid)-2-naphthol-4-sulphonic Acid, Disodium Salt): $C_{20}H_{12}N_2O_{11}S_3Na_2 = 598.50$

General laboratory reagent grade of commerce

Deposited on crystals of sodium chloride in the concentration of about 1 per cent. Use ACS reagent grade.

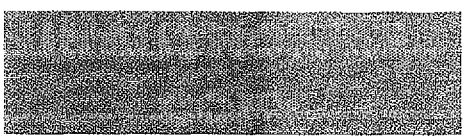
Indophenol Blue; CI 49700: $C_{18}H_{16}N_2O = 276.34$

General laboratory reagent grade of commerce.

Dark purple powder.

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin-layer chromatography* (2.4.17), using *silica gel G* as the coating substance and *dichloromethane* as the mobile phase. Apply to the plate 10 µl of a 0.01 per cent w/v solution in *dichloromethane*. After removal of the plate, allow it to dry in air. The chromatogram shows only one spot, but a stain may remain at the point of origin.



Litmus: Fragments of blue pigment prepared from various species of *Rocella, Lecanora* or other lichens. It has a characteristic odour. Partly soluble in *water* and in *ethanol*.

NOTE — Litmus is unsuitable for determining the pH of alkaloids, carbonates and bicarbonates.

Litmus Solution: Boil 25 g of coarsely powdered *litmus* with 100 ml of *ethanol (90 per cent)* under a reflux condenser for 1 hour and discard the clear liquid. Repeat this operation with two quantities, each of 75 ml of *ethanol (90 per cent)*. Digest the extracted litmus with 250 ml of *water* and filter.

Metacresol Purple; 4,4'-(3*H*-2,1-Benzoxanthiol-3-ylidene)-di-*m*-cresol *S,S*-dioxide: $C_{21}H_{18}O_5S = 382.43$

General laboratory reagent grade of commerce.

***m*-Cresol Purple Solution**: Dissolve 0.1 g of *m*-cresol purple in 13 ml of *0.01M sodium hydroxide*, dilute to 100 ml with *water* and mix.

Metalphthalein; Phthalein Purple: $C_{32}H_{32}N_2O_{12} = 636.6$

Creamy white brown powder.

Complies with the following test.

SENSITIVITY — Dissolve 10 mg in 1 ml of *strong ammonia solution* and dilute to 100 ml with *water*. To 5 ml of the solution add 95 ml of *water*, 4 ml of *strong ammonia solution*, 50 ml of *ethanol (95 per cent)* and 0.2 ml of *0.1 M barium chloride*; the solution is bluish violet. Add 0.24 ml of *0.05 M disodium edetate*; the solution becomes colourless.

Metanil Yellow; CI 13065; Sodium 4-anilinoazobenzene-3-sulphonate: $C_{18}H_{14}N_3NaO_3S = 375.40$

Brownish yellow powder; soluble in *water* and in *ethanol*; slightly soluble in *acetone* and in *ether*.

Metanil Yellow Solution: A 0.1 per cent w/v solution of *metanil yellow* in *methanol*.

Complies with the following test.

SENSITIVITY — Add 0.1 ml of the solution to 50 ml of *anhydrous glacial acetic acid*; the mixture is pinkish red. Add 0.05 ml of *0.1 M perchloric acid*; the colour changes to violet.

Methyl Orange; CI 13025; Sodium 4-dimethylamino-azobenzene-4-sulphonate: $C_{14}H_{14}N_3NaO_3S = 327.34$

Orange-yellow powder or crystalline scales; Sparingly soluble in hot *water*; slightly soluble in *water*; practically insoluble in *ethanol*.

Methyl Orange Solution: Dissolve 0.1 g of *methyl orange* in 80 ml of *water* and add sufficient *ethanol (95 per cent)* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* is yellow. Not more than 0.1 ml of *0.1 M hydrochloric acid* is required to change the colour to red.

Methyl Red; CI 13020; 2-(4-Dimethylaminophenylazo)-benzoic Acid: $C_{15}H_{15}N_3O_2 = 269.30$

Dark red powder or violet crystals; mp, about 182°; soluble in *ethanol*; practically insoluble in *water*.

Methyl Red- Methylene Blue Solution; Methyl Red Mixed Solution: Dissolve 0.1 g of *methyl red* and 50 mg of *methylene blue* in 100 ml of *ethanol (95 per cent)*. Colour changes from reddish violet to green (pH range, 5.2 to 5.6).

Methyl Red Solution: Dissolve 50 mg of *methyl red* in a mixture of 1.86 ml of *0.1 M sodium hydroxide* and 50 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution, 100 ml of *carbon dioxide-free water* and 0.05 ml of *0.02 M hydrochloric acid* is red. Not more than 0.1 ml of *0.02 M sodium hydroxide* is required to change the colour to yellow.

Methylenebisacrylamide. $C_7H_{10}N_2O_2 = 154.2$

A fine, white or almost white powder, slightly soluble in *water*, soluble in *alcohol*.

mp. it melts with decomposition at a temperature above 300°.

Methylene Blue; CI 52015; Basic Blue 9; 3,7-Bis-(dimethyl-amino)phenothiazin-5-ium chloride:

$C_{16}H_{18}ClN_3S,xH_2O = 319.86$ (anhydrous)

Redox indicator grade suitable for biological work.

Dark green or brown crystals with bronze lustre or crystalline powder; hygroscopic; soluble in *water* and in *chloroform*; sparingly soluble in *ethanol*; insoluble in *ether*.

Methylene Blue Solution: Dissolve 150 mg of *methylene blue* in 100 ml of *ethanol (95 per cent)* and dilute with *ethanol (95 per cent)* to produce 250 ml.

Methyl Orange-Xylene Cyanol FF Solution: Dissolve 0.1 g of *methyl orange* and 0.26 g of *xylene cyanol FF* in 50 ml of *ethanol (95 per cent)* and add sufficient *water* to produce 100 ml.

Methyl Thymol Blue; [3*H*-2,1-Benzoxathiol-3-ylidenebis-(6-hydroxy-5-isopropyl-2-methyl-*m*-phenylene) methylene-nitrilo]tetraacetic acid *S, S*-dioxide Tetrasodium salt: $C_{37}H_{40}N_2Na_4O_{13}S = 844.75$

General laboratory reagent grade of commerce.

Produces a blue colour with calcium ions in alkaline solution. When metal ions are absent, for example, in the presence of an excess of disodium edetate, the solution is grey.

1-Naphtholbenzein; α-Naphtholbenzein; Phenyl bis-(4-hydroxynaphthyl)methenol: $C_{27}H_{18}O_2 = 374.43$
Brownish red powder or shiny brownish black crystals.

1-Naphtholbenzein Solution; α -Naphtholbenzein Solution: A 0.2 per cent w/v solution of *1-naphtholbenzein* in *anhydrous glacial acetic acid.*

Complies with the following test.

SENSITIVITY — Add 0.25 ml to 50 ml of *anhydrous glacial acetic acid.* Not more than 0.05 ml of *0.1 M perchloric acid* is required to change the colour of the solution from brownish yellow to green.

Neutral Red; CI 50040; Basic Red 5; 3-Amino-7-dimethylamino-2-methylphenazine Monohydrochloride: $C_{15}H_{16}N_4,HCl = 288.78$

Reddish to olive green coarse powder; sparingly soluble in *ethanol* and in *water.*

Neutral Red Solution: A 0.1 per cent w/v solution of *neutral red* in *ethanol (50 per cent).*

Nile Blue A; CI 51180; 5-Amino-9-diethylaminobenzo[α]-phenoxazinylium Hydrogen Sulphate: $C_{20}H_{21}N_3O_5S = 415.47$

Green,crystalline powder with a bronze lustre.

Complies with the following test.

LIGHT ABSORBTION — A 0.0005 per cent w/v solution in *ethanol (50 per cent)* exhibits a maximum at about 640 nm (2.4.7).

Nile Blue A Solution: A 1 per cent w/v solution of *nile blue A* in *anhydrous glacial acetic acid.*

Complies with the following test.

SENSITIVITY — A solution containing 0.25 ml in 50 ml of *anhydrous glacial acetic acid* is blue. Not more than 0.1 ml of *0.1 M perchloric acid* is required to change the colour of the solution to bluish green.

Colour changes from blue to red (pH range, 9.0 to 13.0).

Oracet Blue B; Solvent Blue 19: A mixture of 1-methylamino-4-anilinoanthraquinone, $C_{21}H_{16}N_2O_2$, and 1-amino-4-anilinoanthraquinone, $C_{20}H_{14}N_2O_2$.

When used for titration in non-aqueous media, it changes from blue (basic) through purple (neutral) to pink (acidic).

Oracet Blue B Solution: A 0.5 per cent w/v solution of *oracet blue B* in *anhydrous glacial acetic acid.*

Phenol Red; Phenolsulphonphthalein; 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)diphenol *S,S*-dioxide:
$C_{19}H_{14}O_5S = 354.39$

Bright to dark red crystalline powder; freely soluble in solutions of alkali carbonates and hydroxides; slightly soluble in *ethanol (95 per cent)*; very slightly soluble in *water.*

Phenol Red Solution: Dissolve 0.1 g of *phenol red* in 2.82 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent).* After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* is yellow. Not more than 0.1 ml of *0.02 M sodium hydroxide* is required to change the colour of the solution to reddish violet.

Phenol Red Reagent:

SOLUTION I — Dissolve 33 mg of *phenol red* in 1.5 ml of *2 M sodium hydroxide* and dilute to 100 ml with *water.*

SOLUTION II — Dissolve 25 mg of *ammonium sulphate* in 235 ml of *water*, add 105 ml of *2 M sodium hydroxide* and 135 ml of *2 M acetic acid.*

Add 25 ml of solution I to solution II. If necessary, adjust the pH of the mixture to 4.7.

Phenolphthalein: Of the Indian Pharmacopoeia.

Phenolphthalein Paper: Immerse strips of filter paper for a few minutes in *phenolphthalein solution, dilute.* Allow to dry.

Phenolphthalein Solution: A 1.0 per cent w/v solution of *phenolphthalein* in *ethanol (95 per cent).*

Phenolphthalein Solution, Dilute: Dissolve 0.1 g of *phenolphthalein* in 80 ml of *ethanol (95 per cent)* and add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* is colourless. Not more than 0.2 ml of *0.02 M sodium hydroxide* is required to change the colour to pink.

Phenolphthalein-Thymol Blue Solution: Dissolve 0.1 g of *thymol blue* in a mixture of 2.2 ml of *0.1 M sodium hydroxide* and 50 ml of *ethanol (95 per cent)* and dilute to 100 ml with *water.* Mix 3 volumes of this solution with 2 volumes of *phenolphthalein solution.*

Pyridylazonaphthol; PAN; 1-(2-Pyridylazo)-2-naphthol:
$C_{15}H_{11}N_3O = 249.27$

General laboratory reagent grade of commerce.

Brick red or orange-red powder; mp, about 140°.

Pyridylazonaphthol Solution: A 0.1 per cent w/v solution in *ethanol.*

Complies with the following test.

SENSITIVITY — To 50 ml of *water* add 10 ml of *acetate buffer pH 4.4*, 0.1 ml of *0.02 M disodium edetate* and 0.25 ml of the

reagent under examination; a yellow colour is produced. Add 0.15 ml of a 0.5 per cent w/v solution of *cupric sulphate*; the colour changes to violet.

Quinaldine Red; 2-(4-Dimethylaminostyryl) quinoline Ethiodide: $C_{21}H_{23}IN_2 = 430.33$

When used for the non-aqueous titration in *anhydrous glacial acetic acid,* the colour changes from magenta (basic) to almost colourless (acidic).

Quinaldine Red Solution: A 0.1 per cent w/v solution of *quinaldine red* in *methanol*.

Ruthenium Red; Ammoniated Ruthenium Oxychloride: $H_{42}Cl_6N_{14}O_2Ru_3,4H_2O = 858.42$

Microscopical staining grade of commerce.

Brownish red powder.

Ruthenium Red Solution: Dissolve 8 mg of *ruthenium red* in 10 ml of *lead acetate solution*.

Silver Manganese Paper: Immerse strips of slow filter paper in a solution containing 0.85 per cent w/v of *manganese (II) sulphate* and 0.85 per cent w/v of *silver nitrate* and leave for a few minutes. Allow the paper to dry over *phosphorus pentoxide* protected from acidic or alkaline vapours.

Sudan Red G; CI 12150; Sudan Red I; 1-(2'-Methoxy-phenylazo)-2-naphthol; $C_{17}H_{14}N_4O = 290.32$

Complies with the following test.

HOMOGENEITY — Carry out the method for *thin-layer chromatography* (2.4.17), using *silica gel G* as the coating substance and *dichloromethane* as the mobile phase but allowing the solvent front to ascend 10 cm above the line of application. Apply to the plate 10 µl of a 0.1 per cent w/v solution in *dichloromethane*. After removal of the plate, allow it to dry in air. The chromatogram shows only one spot.

Thymol Blue; Thymolsulphonphthalein; 4,4'-(3*H*)-2,1-Benzoxathiol-3-ylidene) dithymol *S,S*-dioxide: $C_{27}H_{30}O_5S$ = 466.60

Brownish green, crystalline powder; soluble in *ethanol (95 per cent)* and in dilute alkali solutions; slightly soluble in *water*.

Thymol Blue Solution: Dissolve 0.1 g of *thymol blue* in 2.15 ml of *0.1 M sodium hydroxide* and 20 ml of *ethanol (95 per cent)*. After solution is effected, add sufficient *water* to produce 100 ml.

Complies with the following test.

SENSITIVITY — A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* to which 0.2 ml of *0.02 M sodium hydroxide* has been added is blue. Not more than 0.1 ml of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Thymol Blue Solution, Ethanolic: Dissolve 0.1 g of *thymol blue* in 100 ml of *ethanol (95 per cent)* and filter, if necessary.

Thymolphthalein; 3,3-Bis(4-hydroxy-5-isopropyl-2-mehtylphenyl)phthalide: $C_{28}H_{30}O_4 = 430.55$

White to slightly yellow crystalline powder; soluble in *ethanol (95 per cent)* and in solutions of alkali hydroxides; insoluble in *water*.

Thymolphthalein Solution: A 0.1 per cent w/v solution of *thymolphthalein* in *ethanol (95 per cent)*.

Complies with the following test.

SENSITIVITY — A mixture of 0.05 ml and 100 ml of *carbon dioxide-free water* is colourless. Not more than 0.05 ml of *0.1 M sodium hydroxide* is required to change the colour to blue.

Titan Yellow; CI 19540; Thiazol Yellow; Sodium 2,2-[(diazoamino)di-*p*-phenylene] bis(6-methylbenzothiazole-7-sulphonate): $C_{28}H_{19}N_5Na_2O_6S_4 = 695.71$

Yellowish brown powder.

Titan Yellow Solution: A 0.05 per cent w/v solution of *titan yellow*.

Complies with the following test.

SENSITIVITY — Add 0.1 ml to a mixture of 10 ml of *water*, 0.2 ml of a 0.0101 per cent w/v solution of *magnesium sulphate* and 1.0 ml of *1 M sodium hydroxide*; a pink colour is produced.

Xylene Cyanol FF; Cl 42135 : $C_{25}H_{27}N_2NaO_6S_2 = 538.6$

Blue, ethanol-soluble dye used as a screening agent in *methyl orange-xylene cyanol FF solution*.

Xylenol Orange; [3*H*-2,1-Benzoxathiol-3-ylidenebis-(6-hydroxy-5-methyl-*m*-phenylene)methylenenitrilo]tetra acetic acid *S,S*-dioxide Tetrasodium Salt: $C_{31}H_{28}N_2Na_4O_{13}S = 760.60$

General laboratory reagent grade of commerce.

Reddish brown, crystalline powder; gives a violet colour with mercury, lead, zinc and certain other metal ions, in alkaline solutions. When metal ions are absent, for example in the presence of an excess of disodium edetate, the solution is yellow.

Xylenol Orange Mixture; Xylenol Orange Triturate: Triturate 1 part of *xylenol orange* with 99 parts of *potassium nitrate*.

Complies with the following test.

SENSITIVITY — Add 50 mg to a mixture of 50 ml of *water*, 1 ml of *2 M acetic acid* and 0.05 ml of *lead nitrate solution*. Add sufficient *hexamine* to change the colour from yellow to violet red. Add 0.1 ml of *0.1 M disodium edetate*; the colour changes to yellow.

Xylenol Orange Solution: Mix 0.1 g of *xylenol orange* with 100 ml of *water* and filter, if necessary.

B. Indicators and Indicator Test Papers

Indicator and test papers are strips of filter paper of suitable dimension and grade impregnated with an indicator or a reagent that is sufficiently stable to provide a convenient form of the impregnated substance. Commercial samples of indicator and test papers are available and may be used. Those required in the assays and tests of the Pharmacopoeia may be prepared as described in the following paragraphs.

Treat strong, white filter paper with *hydrochloric acid* and wash with *water* until the last washing does not show an acidic reaction to *methyl red*. Then treat with *dilute ammonia solution* and wash again with *water* until the last washing is not alkaline to *phenolphthalein*. Dry the paper thoroughly and saturate it with the proper strength of the indicator solution or reagent solution and dry carefully in still air by suspending it from glass rods in a space free from acid and other fumes. Cut the paper into strips of suitable size and store in well-closed, light-resistant containers, protected from moisture.

Lead Acetate Paper: Prepare from *lead acetate solution* and dry the impregnated paper at 100°, avoiding contact with metal.

Litmus Paper: Use *red litmus paper* or *blue litmus paper*, as appropriate.

Litmus Paper, Blue: Boil 10 parts of coarsely powdered *litmus* under a reflux condenser for 1 hour with 100 parts of *ethanol (95 per cent)*, decant the *ethanol* and discard. To the residue add a mixture of 45 parts of *ethanol (95 per cent)* and 55 parts of *water*. After 2 days, decant the clear liquid. Impregnate strips of filter paper with the extract and allow to dry.

Complies with the following test.

SENSITIVITY — Immerse a strip, 60 mm x 10 mm, in 100 ml of *0.002 M hydrochloric acid*. On shaking, the paper turns red within 45 seconds.

Litmus Paper, Red: To the extract obtained in the preparation of *blue litmus paper* add *2 M hydrochloric acid* dropwise until the blue solution turns red. Impregnate strips of filter paper with the solution and allow to dry.

Complies with the following test.

SENSITIVITY — Immerse a strip, 60 mm x 10 mm in 100 ml of *0.002 M sodium hydroxide*. On shaking, the paper turns blue within 45 seconds.

Mercuric Chloride Paper: Smooth white filter paper, not less than 25 mm in width, soaked in a saturated solution of *mercuric chloride*, pressed to remove superfluous solution and dried at about 60° in the dark. The grade of filter paper is such that the weight is between 65 and 120 g per sq.m; the thickness in mm of 400 papers is approximately equal, numerically, to the weight in g per sq.m.

Store in a stoppered bottle in the dark. The paper which has been exposed to sunlight or to the vapour of ammonia affords a lighter stain or no stain at all when employed in the *limit test for arsenic* (2.3.10).

Starch Iodate Paper; Starch-iodate Paper: Immerse strips of filter paper in 100 ml of *iodide-free starch solution* containing 0.1 g of *potassium iodate*. Drain and allow to dry protected from light.

Starch Iodide Paper; Starch-iodide Paper: Prepare from a mixture of equal volumes of *starch solution* and 5 per cent w/v solution of *potassium iodide* in *water* and dry the impregnated paper protected from light.

Complies with the following test.

SENSITIVITY — Mix 0.05 ml of *0.1 M sodium nitrite* with 4 ml of *hydrochloric acid* and dilute with *water* to 100 ml. Deposit one drop of the resulting solution on the paper; a blue spot appears.

Titan Yellow Paper: Impregnate filter paper with *titan yellow solution* and dry at room temperature.

4.4. Standard Solutions

Acetaldehyde Standard Solution (100 ppm C_2H_4O): Dissolve 1.0 g of *acetaldehyde* in sufficient *2-propanol* to produce 100 ml and dilute 5.0 ml of the solution to 500.0 ml with *2-propanol*.

Aluminium Standard Solution (2 ppm Al): Dilute 2 volumes of a 0.176 per cent w/v solution of *aluminium potassium sulphate* in 0.1 M *sulphuric acid* to 100 volumes with *water*.

Aluminium Standard Solution (10 ppm Al): Dilute 1 volume of a 1.39 per cent w/v solution of *aluminium nitrate* to 100 volumes with *water*.

Aluminium Standard Solution (200 ppm Al): Dissolve a quantity of *aluminium potassium sulphate* equivalent to 0.352 g of $AlK(SO_4)_2,12H_2O$ in sufficient amount of *water*, add 10.0 ml of *dilute sulphuric acid* and dilute to 100.0 ml with *water*.

Ammonium Standard Solution (1 ppm NH_4): Dilute 10.0 ml of a 0.0741 per cent w/v solution of *ammonium chloride* to 25.0 ml with *ammonia-free water*. Dilute 1 volume of the resulting solution to 100 volumes with *ammonia-free water*, immediately before use.

Ammonium Standard Solution (100 ppm NH_4): Dilute 10 ml of a 0.074 per cent w/v solution of *ammonium chloride* to 25 ml with *water*, immediately before use.

Antimony Standard Solution (100 ppm Sb): Dissolve a quantity of *antimony potassium tartrate* equivalent to 0.274g of $C_4H_4KO_7Sb.½H_2O$ in about 500 ml of *1 M hydrochloric acid* and dilute the clear solution to 1000.0 ml with *water*.

Arsenic Standard Solution (1 ppm As): Dilute 1 volume of *arsenic standard solution (10 ppm As)* to 10 volumes with *water*.

Arsenic Standard Solution (10 ppm As): Dissolve 0.33 g of *arsenic trioxide* in 5 ml of *2 M sodium hydroxide* and dilute to 250.0 ml with *water*. Dilute 1 volume of this solution to 100 volumes with *water*.

Barium Standard Solution (10 ppm Ba): Dilute 1.0 ml of a 0.178 per cent w/v solution of *barium chloride* to 100.0 ml with *water*.

Barium Standard Solution (50 ppm Ba): Dilute 1.0 ml of a 0.178 per cent w/v solution of *barium chloride* to 20.0 ml of *water*, immediately before use

Cadmium Standard Solution (10 ppm Cd): Dilute 2.0 ml of a 0.228 per cent w/v solution of *cadmium sulphate* to 200.0 ml with *water*.

Calcium Standard Solution (10 ppm Ca): Dissolve 0.624 g of dried *calcium carbonate* in *distilled water* containing 3 ml of *5 M acetic acid* and dilute to 250.0 ml with *distilled water*. Dilute 1.0 volume of this solution to 100.0 volumes with *distilled water*.

Calcium Standard Solution (100 ppm Ca), Ethanolic: Dissolve 2.50 g of dried *calcium carbonate* in 12 ml of *5 M acetic acid* and dilute to 1000.0 ml with *distilled water*. Dilute 1.0 volume of this solution to 10.0 volumes with *ethanol* (95 per cent).

Chloride Standard Solution (5 ppm Cl): Dilute 1 volume of a 0.0824 per cent w/v solution of *sodium chloride* to 100 volumes with *water*.

Chloride Standard Solution (25 ppm Cl): Dilute 5 volumes of a 0.0824 per cent w/v solution of *sodium chloride* to 100 volumes with *water*.

Cobalt Standard Solution (100 ppm Co): Dissolve a quantity of *cobalt nitrate* equivalent to 0.494 g of $Co(NO_3)_2,6H_2O$ in about 500 ml of *1 M nitric acid* and dilute the clear solution to 1000.0 ml with *water*.

Copper Standard Solution: Dissolve 1.965 g of *cupric sulphate*, accurately weighed, in sufficient *0.1 M hydrochloric acid* to produce 1000.0 ml. Transfer 3.0 ml to a 1000-ml volumetric flask and dilute to volume with *0.1 M hydrochloric acid*.

1 ml of Copper Standard Solution contains equivalent of 1.5 µg of copper.

Copper Standard Solution (10 ppm Cu): Dilute 1 volume of a 0.393 per cent w/v solution of *cupric sulphate* to 100 volumes with *water*.

Digitoxin Standard Solution: Digitoxin Reagent: Dissolve 0.125 g of digitoxin RS in sufficient *glacial acetic acid* to produce 100.0 ml. Dilute 4.0 ml of this solution to 100.0 ml with *glacial acetic acid*. To 25.0 ml of the resulting solution add 3.0 ml of *water* and mix well.

Digoxin Standard Solution: Dissolve 0.125 g of *digoxin RS* in sufficient *glacial acetic acid* to produce 100.0 ml. Dilute 4.0 ml of this solution to 100.0 ml with *glacial acetic acid*. To 25.0 ml of the resulting solution add 3.0 ml of *water* and mix well.

Ferricyanide Standard Solution (50 ppm $Fe(CN)_6$): Immediately before use, dilute with *water* to 100 times its volume a solution containing *potassium ferricyanide* equivalent to 0.78 g of $K_3Fe(CN)_6$ in 100.0 ml.

Fluoride Standard Solution (10 ppm F). Dissolve a quantity equivalent to 0.442 g of *sodium fluoride* which has been previously dried at 300 ° for 12 hours in *water* and dilute to 1000.0 ml with *water* (1 ml = 0.2 mg F). Store in a polyethylene container. Immediately before use, dilute the solution to 20 times its volume with *water*.

Ferrocyanide Standard Solution (100 ppm $Fe(CN)_6$): Immediately before use, dilute with *water* to 10 times its volume a solution containing *potassium ferrocyanide* equivalent to 0.2 g of $K_4Fe(CN)_6,3H_2O$ in 100.0 ml.

Formaldehyde Standard Solution (5 ppm CH_2O): Dilute 1 volume of a solution containing 3.0 g of *formaldehyde solution* in 1000.0 ml to 200 volumes with *water*.

Germanium Standard Solution (100 ppm Ge): Dissolve a quantity of *ammonium hexafluorogermanate (IV)* equivalent to 0.307 g of $(NH_4)_2GeF_6$ in a 0.01 per cent *v/v* solution of *hydrofluoric acid*. Dilute the clear solution to 1000.0 ml with *water*.

Iron Standard Solution (2 ppm Fe): Dilute 1 volume of *iron standard solution (20 ppm Fe)* to 10 volumes with *water*.

Iron Standard Solution (8 ppm Fe): Dilute 4 volumes of *iron standard solution (20 ppm Fe)* to 10 volumes with *water*.

Iron Standard Solution (10 ppm Fe): Dissolve 7.022 g of *ferrous ammonium sulphate* in *water* containing 25 ml of *1 M sulphuric acid* and add sufficient *water* to produce 1000.0 ml. Dilute 1 volume to 100 volumes with *water*. Contains iron in ferrous state.

Iron Standard Solution (20 ppm Fe): Dilute 1 volume of a 0.1726 per cent w/v *solution of ferric ammonium sulphate* in *0.05 M sulphuric acid* to 10 volumes with *water*. Contains iron in ferric state.

Lead Standard Solution: On the day of use, dilute 10 ml of *lead nitrate stock solution* with *water* to 100.0 ml. 1 ml of *lead standard solution* contains the equivalent of 10 µg of lead. A control comparison solution prepared with 2.0 ml of *lead standard solution* contains, when compared to a solution representing 1.0 g of the substance under examination, the equivalent of 20 ppm of lead.

Lead Standard Solution (1 ppm Pb): Dilute 1 volume of *lead standard solution (10 ppm Pb)* to 10 volumes with *water*.

Lead Standard Solution (2 ppm Pb): Dilute 1 volume of *lead standard solution (10 ppm Pb)* to 5 volumes with *water*.

Lead Standard Solution (10 ppm Pb): Dilute 1 volume of *lead standard solution (100 ppm Pb)* to 10 volumes with *water*.

Lead Standard Solution (20 ppm Pb): Dilute 1 volume of *lead standard solution (100 ppm Pb)* to 5 volumes with *water*.

Lead Standard Solution (100 ppm Pb): Dilute 1 volume of *lead standard solution (0.1 per cent Pb)* to 10 volumes with *water*.

Lead Standard Solution (0.1 per cent Pb): Dissolve 0.4 g of *lead nitrate* in *water* containing 2 ml of *nitric acid* and add sufficient *water* to produce 250.0 ml.

Manganese Standard Solution (100 ppm Mn): Dissolve a quantity of *manganese sulphate* equivalent to 0.308g of $MnSO_4,H_2O$ in about 500ml of *1M nitric acid* and dilute the clear solution to 1000.0 ml with *water*.

Mercury Standard Solution (100 ppm Hg) Dissolve 0.108 g of *yellow mercuric oxide* in the minimum volume of *2 M hydrochloric acid*, add sufficient *water* to produce 1000.0 ml.

Nickel Standard Solution (10 ppm Ni): Dilute 1 volume of a 0.478 per cent w/v solution of *nickel sulphate* to 100 volumes with *water*.

Nitrate Standard Solution (2 ppm NO_3): Dilute 1 volume of *nitrate standard solution (100 ppm NO_3)* to 50 volumes with *water*.

Nitrate Standard Solution (100 ppm NO_3): Dilute 1 volume of a 0.163 per cent w/v solution of *potassium nitrate* to 10 volumes with *water*.

Nitrite Standard Solution (20 ppm NO_2). Dissolve 0.6 g of *sodium nitrite* in 100 ml of *water* and dilute 1 ml of this solution to 200 ml with *water*.

Palladium Standard Solution (20 ppm Pd): Dissolve 0.333 g of *palladium chloride* in 2 ml of warm *hydrochloric acid*. Dilute the solution to 1000 ml with a mixture of equal volumes of *dilute hydrochloric acid* and *water*. Immediately before use dilute to 10 times its volume with *water*.

Phosphate Standard Solution (5 ppm PO_4): Dilute 1 volume of a 0.143 per cent w/v solution of *potassium dihydrogen phosphate* to 200 volumes with *water*.

Silver Standard Solution (5 ppm Ag): Dilute 1 volume of a 0.079 per cent w/v solution of *silver nitrate* to 100 volumes with *water*.

Strontium Standard Solution (1.0 per cent Sr): Cover with *water, strontium carbonate* equivalent to 1.6849 g of $SrCO_3$. Cautiously add *hydrochloric acid* until the entire solid has dissolved and there is no sign of further effervescence. Dilute to 100.0 ml with *water*.

Sulphate Standard Solution (10 ppm SO_4): Dilute 1 volume of a 0.181 per cent w/v solution of *potassium sulphate* in *distilled water* to 100 volumes with the same solvent.

Sulphate Standard Solution (10 ppm SO_4), Ethanolic: Dilute 1 volume of a 0.181 per cent w/v solution of *potassium sulphate* in *ethanol (30 per cent)* to 100 volumes with *ethanol (30 per cent)*.

Tin Standard Solution (5 ppm Sn): Dissolve 0.5 g of *tin* in a mixture of 5 ml of *water* and 25 ml of *hydrochloric acid* and add sufficient *water* to produce 1000.0 ml. Dilute 1 volume of this solution to 100 volumes with a 2.5 per cent v/v solution of *hydrochloric acid*.

Titanium Standard Solution (100 ppm Ti): Dissolve a quantity of 100 mg of *titanium* in about 100 ml of *hydrochloric acid* diluted to 150 ml with *water*, heating if necessary. Allow to cool and dilute to 1000.0 ml with *water*.

Zinc Standard Solution (10 ppm Zn): Dilute 1 volume of *zinc standard solution (100 ppm Zn)* to 10 volumes with *water*.

Zinc Standard Solution (25 ppm Zn): Dilute 25 volumes of *zinc standard solution (100 ppm Zn)* to 100 volumes with *water*.

Zinc Standard Solution (100 ppm Zn): Dissolve 0.440 g of *zinc sulphate* in *water* containing 1 ml of 5M acetic acid and add sufficient *water* to produce 100.0 ml.

Dilute 1 volume of this solution to 10 volumes with *water* immediately before use.

4.5. Volumetric Reagents and Solutions

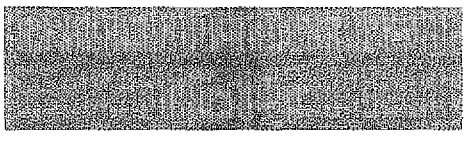
Volumetric solutions, also known as standard solutions, are solutions of reagents of known concentrations intended primarily for use in quantitative determinations. Concentrations are usually expressed in terms of *molarity (M)*.

Molar Solutions

A molar solution contains 1 g molecule of the reagent in 1000 ml of the solution. Thus, each litre of a molar solution of *sodium nitrite* contains 69.0 g of $NaNO_2$ and each litre of a molar solution of *disodium edetate* contains 372.2 g of $C_{10}H_{14}N_2Na_2O_8,2H_2O$. Solutions containing one-tenth of a gram-molecule of the reagent in 1000 ml are designated as 'tenth-molar' or 0.1M; other molarities are similarly indicated.

Preparation and Standardisation of Volumetric Solutions

It is not always possible nor is it essential, to prepare volumetric solutions of a desired theoretical molarity. A solution of approximately the desired molarity is prepared and standardised by titration against a solution of a primary standard. The molarity factor so obtained is used in all calculations, where such standardised solutions are employed. As the strength of a standard solution may change upon



standing, the molarity factor should be redetermined frequently. Volumetric solutions should not differ from the prescribed strength by more than 10 per cent and the molarity should be determined with a precision of 0.5 per cent.

When solutions of a reagent are used in several molarities, the details of the preparation and standardisation are usually given for the most commonly used strength. Stronger or weaker solutions are prepared and standardised using proportionate amounts of the reagent or by making an exact dilution of a stronger solution. Volumetric solutions prepared by dilution should be restandardised either as directed for the stronger solution or by comparison with another volumetric solution having a known ratio to the stronger solution.

The water used in preparing volumetric solutions complies with the requirements of the monograph on Purified Water, unless otherwise specified. When used for the preparation of unstable solutions such as potassium permanganate or sodium thiosulphate, it should be freshly boiled and cooled. When a solution is to be used in an assay in which the end-point is determined by an electrochemical process (e g. potentiometrically), the solution must be standardised in the same way.

Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination should be done using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under examination but omitting the substance under examination.

Primary Standards

These are materials which, after drying under the specified conditions, are recommended for use as primary standards in the standardisation of volumetric solutions. The following are recommended for use as primary standards.

Arsenic Trioxide: Sublime *arsenic trioxide* in an appropriate apparatus and store over *silica gel.*

Benzoic Acid: Sublime *benzoic Acid* in an appropriate apparatus and store in a tightly- closed container.

Potassium Bromate: Recrystallise *potassium bromate* from boiling *water*. Collect the crystals and dry to constant weight at 180°. Store in a tightly-closed container.

Potassium Dichromate: Heat *potassium dichromate* to 140° to150°in an oven, cool in a desiccator and powder in a glass mortar.

Potassium Hydrogen Phthalate: Recrystallise *potassium hydrogen phthalate* from boiling *water*, collect the crystals at a temperature above 35°and dry to constant weight at 110°. Store in a tightly-closed container.

Potassium Iodate: Recrystallise *potassium iodate* from boiling *water*. Collect the crystals and dry to constant weight at 120°. Store in tightly-closed container.

Sodium Carbonate, Anhydrous: Filter at room temperature a saturated solution of *sodium carbonate*. Introduce slowly into the filtrate a stream of *carbon dioxide*, with constant cooling and stirring. After about 2 hours, collect the precipitate on a sintered glass filter. Wash the filter with ice-cold *water* saturated with *carbon dioxide*. After drying at 100° to105°, heat to constant weight at 270° to 300°, stirring from time to time. Store in a tightly-closed container.

Sodium Chloride: To 1 volume of a saturated solution of *sodium chloride* add 2 volumes of *hydrochloric acid*. Collect the crystals formed and wash with *hydrochloric acid*. Remove the hydrochloric acid by heating on a water-bath and dry the crystals to constant weight at 300°. Store protected from moisture.

Sulphanilic Acid: Recrystallise *sulphanilic acid* from boiling *water*. Filter and dry to constant weight at 100° to 105°.

Zinc, Granulated: Wash *granulated zinc* with *dilute hydrochloric acid*, followed by *water*, *ethanol (95 per cent)* and finally *acetone*. Dry at 100° for 5 minutes and cool in a desiccator over *silica gel*.

Volumetric Solutions

Ammonium Thiocyanate, 0.1 M: Dissolve 7.612 g of *ammonium thiocyanate* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Pipette 30.0 ml of *0.1 M silver nitrate* into a glass-stoppered flask, dilute with 50 ml of *water*, add 2 ml of *nitric acid* and 2 ml of *ferric ammonium sulphate solution* and titrate with the *ammonium thiocyanate solution* to the first appearance of a red-brown colour.

1 ml of *0.1 M silver nitrate* is equivalent 0.007612 g of NH_4SCN.

Barium Chloride, 0.05 M: Dissolve 12.2 g of *barium chloride* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

To 10.0 ml of the solution add 60 ml of *water*, 3 ml of *strong ammonia solution* and 0.5 to 1 mg of *metalphthalein* as indicator and titrate with *0.05 M disodium edetate*. As the solution begins to decolorise, add 50 ml of *ethanol (95 per cent)* and titrate until the bluish violet colour is discharged.

1 ml of *0.05 M disodium edetate* is equivalent to 0.012215 g of $BaCl_2,2H_2O$.

Barium Perchlorate, 0.025 M: Dissolve 15.8 g of *barium hydroxide* in a mixture of 75 ml of *water* and 7.5 ml of *perchloric acid*, adjust to pH 3.0 with *perchloric acid* and filter if necessary. To the solution add 150 ml of *ethanol (95 per cent)*, dilute to 250 ml with *water* and add sufficient

buffer solution pH 3.7 to produce 1000 ml. Dilute 500 ml of this solution to 1000 ml with *buffer solution pH 3.7*. Standardise the solution in the following manner.

To 5.0 ml of *0.05 M sulphuric acid* add 5.0 ml of *water*, 50 ml of *acetate buffer pH 3.7* and 0.5 ml of *alizarin Red S solution* and titrate with the *barium perchlorate solution* until an orange-red colour appears.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.01681 g of $Ba(ClO_4)_2$.

Benzethonium Chloride, 0.004 M: Dissolve 1.792 g of *benzethonium chloride*, previously dried to constant weight at 105°, in sufficient *water* to produce 1000 ml. Standardise the solution from the content of $C_{27}H_{42}ClNO_2$ in the dried benzethonium chloride determined in the following manner.

Dissolve 0.35 g of the dried substance in 30 ml of *anhydrous Glacial acetic acid*, add 6 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid* using 0.05 ml of *crystal violet solution* as indicator. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04481 g of $C_{27}H_{42}ClNO_2$.

Bromine, 0.05 M: Dissolve 3 g of *potassium bromate* and 15 g of *potassium bromide* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Pipette 25.0 ml of the solution into a 500 - ml iodine flask and dilute with 120 ml of *water*. Add 5 ml of *hydrochloric acid*, insert the stopper in the flask and shake it gently. Add 5 ml of *potassium iodide solution*, again insert the stopper and allow it to stand for 5 minutes in the dark. Titrate the liberated iodine with *0.1 M sodium thiosulphate* using 3 ml of *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.01598 g of Br_2.

Store in dark amber-coloured, glass stoppererd bottles.

Ceric Ammonium Nitrate, 0.1 M; Ammonium Ceric Nitrate, 0.1 M: Dissolve 54.82 g of *ceric ammonium nitrate* in 1000 ml of *1 M nitric acid* and filter. Standarised the solution as described under *0.1 M ceric ammonium sulphate*.

1 ml of *0.1 M ceric ammonium nitrate* is equivalent to 0.0067 g of $Na_2C_2O_4$.

Store protected from light.

Ceric Ammonium Sulphate, 0.1 M; Ammonium Ceric Sulphate, 0.1 M: Dissolve 65 g of *ceric ammonium sulphate*, with the aid of gentle heat, in a mixture of 30 ml of *sulphuric acid* and 500 ml of *water*. Cool, filter the solution, if turbid, and dilute to 1000 ml with *water*. Standardise the solution in the following manner.

Weigh and transfer accurately about 0.2 g of *sodium oxalate*, previously dried at 105° for 1 hour, to a 250 ml conical flask. Dissolve in 100 ml of *water*, add 2 ml of *sulphuric acid*, mix well, add 10 ml of *hydrochloric acid*, mix well and heat to about 75°. Titrate with 0.1 M *ceric ammonium sulphate* until the solution becomes faintly yellow.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.0067 g of $Na_2C_2O_4$.

Ceric Ammonium Sulphate, 0.01 M: To 100 ml of *0.1 M ceric ammonium sulphate* add, while cooling, 30 ml of *sulphuric acid* and sufficient *water* to produce 1000 ml.

Cupric Sulphate, 0.02 M: Dissolve 5.0 g of *cupric sulphate* in *water* and dilute to 1000 ml with *water*. Standardise the solution in the following manner.

To 20.0 ml add 2 g of *sodium acetate* and titrate with *0.02 M disodium edetate*, using 0.1 ml of *pyridylazonaphthol solution* as indicator, until the colour changes from violet-blue to bright green, adding the titrant slowly towards the end-point.

1 ml of *0.02 M disodium edetate* is equivalent to 0.004994 g of $CuSO_4,5H_2O$.

Dimethyl Glutaric Acid, 0.005 M: Dissolve 80 mg of *dimethyl glutaric acid* in 90 ml of *water* in 100 ml volumetric flak, adjust the pH to 7.0 with 1 M *sodium hydroxide* and make up the volume with *water*.

Dioctyl Sodium Sulphosuccinate, 0.005 M: Dissolve 2.25 g of *dioctyl sodium sulphosuccinate* in warm *water*, cool and dilute to 1000 ml with *water*. Standardise the solution in the following manner.

To 25.0 ml add 25 ml of a solution containing 20 per cent w/v of *anhydrous sodium sulphate* and 2 per cent w/v of *sodium carbonate*, 50 ml of *chloroform* and 1.5 ml of *bromophenol blue solution* and mix. Titrate with *0.01 M tetrabutylammonium iodide* until about 1 ml from the end point. Stopper the flask, shake vigorously for 2 minutes and continue the titration, in increments of 0.05 ml, shaking vigorously and allowing the flask to stand for about 10 seconds after each addition. Continue the titration until a blue colour just appears in the chloroform layer.

1 ml of *0.01M tetrabutylammonium iodide* is equivalent to 0.004446 g of $C_{20}H_{37}NaO_7S$.

Disodium Edetate, 0.1 M: Dissolve 37.2 g of *disodium edetate* in sufficient *water to* produce 1000 ml. Standardise the solution in the following manner.

Weigh accurately about 0.8 g of *granulated zinc*, dissolve by gentle warming in 12 ml of *dilute hydrochloric acid* and 0.1 ml of *bromine water*. Boil to remove excess bromine, cool and add sufficient *water* to produce 200.0 ml. Pipette 20.0 ml of the resulting solution into a flask and nearly neutralise with

2 M sodium hydroxide. Dilute to about 150 ml with *water*, add sufficient *ammonia buffer pH 10.0* to dissolve the precipitate and add 5 ml in excess. Add 50 mg of *mordant black II mixture* and titrate with the *disodium edetate solution* until the solution turns green.

1 ml of *0.1 M disodium edetate* is equivalent to 0.00654 g of Zn.

Ferric Ammonium Sulphate, 0.1 M: Ammonium Iron(III) Sulphate, 0.1M: Dissolve 50 g of *ferric ammonium sulphate* in a mixture of 300 ml of *water* and 6 ml of *sulphuric acid* and dilute with sufficient freshly boiled and cooled *water* to produce 1000 ml. Standardise the solution in the following manner.

To 25.0 ml add 3 ml of *hydrochloric acid* and 2 g of *potassium iodide*, allow to stand for 10 minutes and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.04822 g of $FeNH_4(SO_4)_2,12H_2O$.

Ferrous Ammonium Sulphate, 0.1 M; Ammonium Iron(II) Sulphate, 0.1 M: Dissolve 40 g of *ferrous ammonium sulphate* in a previously cooled mixture of 40 ml of *sulphhuric acid* and 200 ml of *water*, dilute with sufficient freshly boiled and cooled *water* to produce 1000 ml. Standardise the solution in the following manner.

Measure accurately 25.0 ml of the solution into a flask, add 2 drops of *1,10-phenanthroline solution* and titrate with *0.1 M ceric ammonium sulphate* until the red colour is changed to pale blue.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.03921g of $Fe(NH_4)_2(SO_4)_2, 6H_2O$.

Hydrochloric Acid, 1 M: Dilute 85 ml of *hydrochloric acid* with *water* to produce 1000 ml. Standardise the solution in the following manner.

Weigh accurately about 1.5 g of *anhydrous sodium carbonate*, previously heated at about 270° for 1 hour. Dissolve it in 100 ml of *water* and add 0.1 ml of *methyl red solution*. Add the acid slowly from a burette, with constant stirring, until the solution becomes faintly pink. Heat the solution to boiling, cool and continue the titration. Heat again to boiling and titrate further as necessary until the faint pink colour is no longer affected by continued boiling.

1 ml of *1 M hydrochloric acid* is equivalent to 0.05299 g of Na_2CO_3.

Hydrochloric Acid, 0.5 M Methanolic: Take 40 ml of *water* in a 1000 ml volumetric flask and slowly add 43 ml of *hydrochloric acid*. Cool and add *methanol* to volume. Standardise the solution in the following manner.

Weigh accurately about 800 mg of *anhydrous sodium carbonate*, previously heated at about 270° for 1 hour, and proceed as directed under *1 M hydrochloric acid*.

Iodine, 0.05 M: Dissolve about 14 g of *iodine* in a solution of 36 g of *potassium iodide* in 100 ml of *water*, add three drops of *hydrochloric acid* and dilute with *water* to 1000 ml. Standardise the solution in the following manner.

Weigh accurately about 0.15 g of *arsenic trioxide*, previously dried at 105° for 1 hour, and dissolve in 20 ml of *1 M sodium hydroxide* by warming, if necessary. Dilute with 40 ml of *water*, add 0.1 ml of *methyl orange solution* and add dropwise *dilute hydrochloric acid* until the yellow colour is changed to pink. Add 2 g of *sodium carbonate*, dilute with 50 ml of *water* and add 3 ml of *starch solution*. Titrate with the *iodine solution* until a permanent blue colour is produced.

1 ml of *0.05 M iodine* is equivalent to 0.004946 g of As_2O_3.

Store in amber-coloured, glass stoppered bottles.

Lead Nitrate, 0.1 M: Dissolve 33.12 g of *lead nitrate* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Pipette 50.0 ml of the solution into a flask, add 50 mg of *xylenol orange mixture* and sufficient *hexamine* to produce a violet-pink colour and titrate with *0.1 M disodium edetate* to a lemon-yellow end point.

1 ml of *0.1 M disodium edetate* is equivalent to 0.03312 g of $Pb(NO_3)_2$.

Lithium Methoxide, 0.1 M: Dissolve in small portions 0.7 g of freshly cut *lithium* in 150 ml of *anhydrous methanol*, cooling the flask during the addition of the metal. When reaction is complete add sufficient *toluene* to produce 1000 ml. If cloudiness or precipitation occurs, add sufficient *anhydrous methanol* to clarify the solution. Standardise the solution immediately before use in the following manner.

Weigh accurately about 0.25 g of *benzoic acid*, dissolve in 25 ml of *dimethylformamide*. Titrate with *lithium methoxide* solution, using *quinaldine red solution* as the indicator and protecting the solution from atmospheric carbon dioxide throughout the titration. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M lithium methoxide* is equivalent to 0.01221 g of $C_7H_6O_2$.

Store the solution in a manner suitably protected from carbon dioxide and moisture.

Magnesium Sulphate, 0.05 M: Dissolve 12.5 g of *magnesium sulphate* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Pipette 30.0 ml of solution into a flask, add 20 ml of *water*, 10 ml of *strong ammonia-ammonium chloride* solution and 50 mg

of *mordant black 11* mixture . Titrate with *0.1 M disodium edetate* until the colour changes from violet to full blue. 1 ml of *0.1 M disodium edetate* is equivalent to 0.02465 g of $MgSO_4,7H_2O$.

Mercuric Nitrate, 0.02 M: Dissolve 6.85 g of *mercuric nitrate* in 20 ml of *1 M nitric acid* and add sufficient *water* to produce 1000 ml.Standardise the solution in the following manner.

Dissolve 15 mg of *sodium chloride* in 50 ml of *water* and titrate with the *mercuric nitrate solution* determining the end-point potentiometrically, using a platinum or mercury indicator electrode and a mercury-mercurous sulphate reference electrode.

1 ml of *0.02 M mercuric nitrate* is equivalent to 0.002338 g of NaCl.

Nitric Acid, 1 M: Dilute 63 ml of *nitric acid* with sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Dissolve 2 g of *anhydrous sodium carbonate* in 50 ml of *water* and titrate with the *nitric acid solution* using *methyl orange solution* as indicator until the solution becomes reddish yellow. Boil for 2 minutes, cool and continue the titration until the reddish yellow colour is restored.

1 ml of *1 M nitric acid* is equivalent to 0.05299 g of Na_2CO_3.

Perchloric Acid, 0.1 M: Mix 8.5 ml of *perchloric acid* with 500 ml of *anhydrous glacial acetic acid* and 25 ml of *acetic anhydride*, cool and add *anhydrous glacial acetic acid* to produce 1000 ml. Allow the prepared solution to stand for 1 day for the excess *acetic anhydride* to be combined and carry out the determination of water (2.3.43). If the *water* content exceeds 0.5 per cent , add more *acetic anhydride*. If the solution contains no titratable water, add sufficient *water* to obtain a content of *water* between 0.02 per cent and 0.5 per cent. Allow the solution to stand for 1 day and again titrate the *water* content. The solution so obtained should contain between 0.02 per cent and 0.5 per cent of water. Standardise the solution in the following manner.

Weigh accurately about 0.35 g of *potassium hydrogen phthalate*, previously powdered lightly and dried at 120° for 2 hours and dissolve it in 50 ml of *anhydrous glacial acetic acid*. Add 0.1 ml of *crystal violet solution* and titrate with the perchloric acid solution until the violet colour changes to emerald-green. Peform a blank determination and make any necessary correction.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02042 g of $C_8H_5KO_4$.

Other strengths of perchloric acid should be prepared by diluting *0.1 M perchloric acid* appropriately with *anhydrous glacial acetic acid*.

In the tests and assays of the Pharmacopoeia, this solution is specified as "*0.1 M perchloric acid*". Thus the solution in *anhydrous glacial acetic acid* is to be used unless the words "in *dioxan*" are stated.

Potassium Dichromate, 0.0167 M: Weigh 4.9 g of *potassium dichromate*, previously powdered and dried in a desiccator for 4 hours, and dissolve in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

To 20.0 ml of the solution add 1 g of *potassium iodide* and 7 ml of *2 M hydrochloric acid*. Add 250 ml of *water* and titrate with *0.1 M sodium thiosulphate*, using 3 ml of *starch solution*, added towards the end point of the titration, as indicator until the colour changes from blue to light green.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.0049 g of $K_2Cr_2O_7$.

Potassium Hydrogen Phthalate, 0.05 M: Dissolve 10.21 g of *potassium hydrogen phthalate* in about 800 ml of *anhydrous glacial acetic acid*, heat on a water-bath until completely dissolved, protected from humidity, cool to 20° and add sufficient *anhydrous glacial acetic acid* to produce 1000 ml.

Potassium Hydroxide, 0.1 M: Dissolve about 6 g of *potassium hydroxide* in sufficient *carbon dioxide free water* to produce 1000 ml. Standardise the solution in the following manner.

Titrate 20.0 ml of the solution with *0.1 M hydrochloric acid* using 0.5 ml of *phenolphthalein solution* as indicator.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.005611 g of KOH.

Potassium Hydroxide, 0.1 M Ethanolic: Dissolve about 6 g of *potassium hydroxide* in 5 ml of *water* and add sufficient *aldehyde-free ethanol (95 per cent)* to produce 1000 ml. Allow the solution to stand in a tightly-stoppered bottle for 24 hours. Then quickly decant the clear supernatant liquid into a suitable, tightly-closed container and standardise the solution in the following manner.

Titrate 20.0 ml of the solution with *0.1 M hydrochloric acid* using 0.5 ml of *phenolphthalein solution* as indicator.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.005611 g of KOH.

Store protected from light and moisture.

Potassium Hydroxide in Ethanol (60 per cent), 0.5 M: Dissolve 30 g of *potassium hydroxide* in sufficient *ethanol (60 per cent)* to produce 1000 ml. Standardise the solution in the following manner.

Titrate 20.0 ml of the solution with *0.5 M hydrochloric acid* using 0.5 ml of *phenolphthalein solution* as indicator.

1 ml of *0.5 M hydrochloric acid* is equivalent to 0.02806 g of KOH.

Potassium Iodate, 0.05 M: Weigh accurately 10.7 g of *potassium iodate*, previously dried at 110° to constant weight, in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Dilute 25.0 ml of the solution to 100 ml with *water* and to 20.0 ml of this solution add 2 g of *potassium iodide* and 10 ml of *1 M sulphuric acid*. Titrate with *0.1 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator and continue to the absence of the blue-starch-iodine complex.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003566 g of KIO_3.

Potassium Permanganate, 0.02 M: Dissolve 3.2 g of *potassium permanganate* in 1000 ml of *water*, heat on a water-bath for 1 hour, allow to stand for 2 days and filter through glass wool. Standardise the solution in the following manner.

To 25.0 ml of the solution in a glass-stoppered flask add 2 g of *potassium iodide*, followed by 10 ml of *1 M sulphuric acid*. Titrate the liberated iodine with *0.1 M sodium thiosulphate*, using 3 ml of *starch solution*, added towards the end of the titration, as indicator. Perform a blank determination and make necessary correction.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003161 g of $KMnO_4$.

Store protected from light.

Silver Nitrate, 0.1 M: Dissolve 17.0 g in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Weigh accurately about 0.1 g of *sodium chloride*, previously dried at 110° for 2 hours and dissolve in 5 ml of *water*. Add 5 ml of *acetic acid*, 50 ml of *methanol* and 0.15 ml of *eosin solution*. Stir, preferably with magnetic stirrer, and titrate with the *silver nitrate solution* until permanent pink precipitate is produced.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Store protected from light.

Sodium Dodecyl Sulphate, 0.001 M: Dissolve 0.2884 g of *sodium dodecyl sulphate*, calculated with reference to the substance dried at 105° for 2 hours, in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

To 50.0 ml add 15 ml of *chloroform*, 10 ml of *1 M sulphuric acid* and 1 ml of *dimethyl yellow-oracet blue solution* and titrate with *0.004 M benzethonium chloride*, shaking vigorously and allowing the layers to separate after each addition, until the chloroform layer acquires a permanent clear green colour.

1 ml of *0.004 M benzethonium chloride* is equivalent to 0.001154 g of $C_{12}H_{25}NaO_4S$.

Sodium Hydroxide, 1 M: Dissolve 42 g of *sodium hydroxide* in sufficient *carbon dioxide-free water* to produce 1000 ml. Standardise the solution in the following manner.

Weigh accurately about 5 g of *potassium hydrogen phthalate*, previously powdered and dried at 120° for 2 hours, and dissolve in 75 ml of *carbon dioxide-free water*. Add 0.1 ml of *phenolphthalein solution* and titrate with the *sodium hydroxide solution* until a permanent pink colour is produced. 1 ml of *1 M sodium hydroxide* is equivalent to 0.2042 g of $C_8H_5KO_4$.

Store in bottles with well-fitted suitable stoppers which prevent access to atmospheric carbon dioxide.

Volumetric solutions of *sodium hydroxide* must be restandardise frequently. Solutions of lower concentrations are prepared by quantitatively diluting accurately measured volumes of *1 M sodium hydroxide* with sufficient *carbon dioxide-free water* to give the desired concentration.

Sodium Hydroxide, 0.1 M Ethanolic: Dissolve 4.2 g of *sodium hydroxide* in 5 ml of *water* and add sufficient *aldehyde-free ethanol* to produce 1000 ml. Allow the solution to stand in a tightly-stoppered bottle for 24 hours. Then quickly decant the clear supernatant liquid into a suitable, tightly-closed container. Standardise the solution in the following manner.

Weigh accurately about 0.6 g of *benzoic acid*, dissolve in a mixture of 30 ml of *ethanol (95 per cent)* and 6 ml of *water* and titrate with the *ethanolic sodium hydroxide solution*, using 0.2 ml of *phenolphthalein solution* as indicator until a permanent pale pink colour is produced.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.01221 g of $C_7H_6O_2$.

Store protected from light and moisture.

Sodium Methoxide, 0.1 M: Cool 150 ml of *anhydrous methanol* in ice *water* and add, in small portions, about 2.5 g of freshly cut *sodium*. When the metal has dissolved, add sufficient *toluene*, previously dried over sodium wire, to produce 1000 ml. Standardise the solution in the following manner immediately before use.

Weigh accurately about 0.4 g of *benzoic acid*, dissolve in 80 ml of *dimethylformamide*, add 0.15 ml of *thymolphthalein solution* and titrate with *sodium methoxide solution* to a blue end-point. Protect the solution from atmospheric carbon dioxide throughout the titration. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M sodium methoxide* is equivalent to 0.01221 g of $C_7H_6O_2$.

Store protected from carbon dioxide and moisture.

Sodium Nitrite, 0.1 M: Dissolve 7.5 g of *sodium nitrite* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Dissolve 0.3 g of *sulphanilic acid* in 50 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and titrate with the *sodium nitrite solution* determining the end-point potentiometrically.

1 ml of *0.1 M sodium nitrite* is equivalent to 0.01732 g of $C_6H_7NO_3S$.

Sodium Thiosulphate, 0.1 M: Dissolve 25 g of *sodium thiosulphate* and 0.2 g of *sodium carbonate* in *carbon dioxide-free water* and dilute to 1000 ml with the same solvent. Standardise the solution in the following manner.

Dissolve 0.200 g of *potassium bromate*, weighed accurately, in sufficient *water* to produce 250.0 ml. To 50.0 ml of this solution add 2 g of *potassium iodide* and 3 ml of *2 M hydrochloric acid* and titrate with the *sodium thiosulphate solution* using *starch solution*, added towards the end of the titration, as indicator until the blue colour is discharged.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.002784 g of $KBrO_3$.

Re-standardise the solution frequently.

Sulphuric Acid, 0.5 M: Add slowly, with stirring, 28 ml of *sulphuric acid* to about 1000 ml of *water*, allow to cool 25° and standardise against *anhydrous sodium carbonate* as described under *1 M hydrochloric acid*.

1 ml of *0.5 M sulphuric acid* is equivalent to 0.05299 g of Na_2CO_3.

Sulphuric Acid, 0.25 M Ethanolic: Add slowly, with stirring, 13.9 ml of *sulphuric acid* to a sufficient quantity of *ethanol* to produce 1000 ml. Cool and standardise against *anhydrous sodium carbonate* as described under *0.5 M methanolic hydrochloric acid*.

Tetrabutylammonium Hydroxide, 0.1 M: Dissolve 40 g of *tetrabutylammonium iodide* in 90 ml of *dehydrated methanol* in a glass-stoppered flask. Place in an ice-bath, add 20 g of powdered *silver oxide*, insert the stopper and agitate vigorously for 1 hour. Centrifuge a few ml, and test the supernatant liquid for iodides (2.3.1). If the test is positive, add an additional 2 g of *silver oxide* and continue to stand for 30 minutes with intermittent agitation. When all of the iodide has reacted, filter through fine sintered-glass filter. Rinse the flask and filter with three quantities, each of 50 ml, of *anhydrous toluene*. Add the washings of the filtrate and dilute to 1000 ml with *anhydrous toluene*. Flush the solution for 10 minutes with dry, carbon dioxide-free nitrogen. Store protected from carbon dioxide and moisture, and discard after 60 days.

Alternatively, prepare the solution by diluting a suitable volume of commercially available *tetrabutylammonium hydroxide solution* in *methanol* with a mixture of four volumes of *anhydrous toluene* and 1 volume of *dehydrated methanol*. Standardise the solution in the following manner immediately before use.

Weigh accurately about 0.4 g of *benzoic acid*, dissolve in 80 ml of *dimethylformamide*, add a few drops of a 1 per cent w/v solution of *thymol blue* in *dimethylformamide* and titrate with the *tetrabutylammonium hydroxide solution* to a blue end-point. Protect the solution from atmospheric carbon dioxide throughout the titration. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.01221 g of $C_7H_6O_2$.

Tetrabutylammonium hydroxide in 2-propanol, 0.1 M: Tetrabutylammonium Hydroxide in propan-2-ol, 0.1 M: Dissolve 40 g of *tetrabutylammonium iodide* in 90 ml of *dehydrated methanol* in a glass-stoppered flask. Place in an ice-bath, add 20 g of powdered *silver oxide*, insert the stopper, and agitate vigorously for 1 hour. Centrifuge a few ml, and test the supernatant liquid for iodides (2.3.1). If the test is positive, add an additional 2 g of *silver oxide* and continue to stand for 30 minutes with intermittent agitation. When all of the iodide has reacted, filter through fine sintered-glass filter. Rinse flask and filter with three quantities, each of 50 ml, of *propan-2-ol*. Add the washings of the filtrate and dilute to 1000 ml with *propan-2-ol*. Flush the solution for 10 minutes with dry, carbon dioxide-free nitrogen. Store protected from carbon dioxide and moisture, and discard after 60 days.

Alternatively, prepare the solution by diluting a suitable volume of commercially available *tetrabutylammonium hydroxide solution in methanol* with a mixture of four volumes of *propan-2-ol* and 1 volume of *dehydrated methanol*.

Standardise the solution in the following manner immediately before use.

Weigh accurately about 0.4 g of *benzoic acid*, dissolve in 80 ml of *dimethylformamide*, add a few drops of a 1 per cent w/v solution of *thymol blue* in *dimethylformamide* and titrate with the *tetrabutylammonium hydroxide solution* to a blue end-point. Protect the solution from atmospheric carbondioxide throughout the titration. Perform a blank determination and make any necessary correction.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.01221 g of $C_7H_6O_2$.

Tetrabutylammonium Iodide, 0.01M: Dissolve 4 g of *tetrabutylammonium iodide* in *water* and dilute to 1000 ml with *water*. Standardise the solution in the following manner.

Pipette 25.0 ml of the solution into a flask, add 50.0 ml of *0.01 M silver nitrate solution*, add 1.0 ml of *1 M nitric acid* and titrate the excess of silver nitrate with *0.01 M ammonium thiocyanate* using 1 ml of *ferric ammonium sulphate solution* as an indicator.

1 ml of *0.01 M silver nitrate* is equivalent to 0.003694 g of $C_{16}H_{37}IN$.

Titanium Trichloride, 0.1 M: Dilute 100 ml of *titanium trichloride solution* with 200 ml of *hydrochloric acid* and add sufficient freshly boiled and cooled *water* to produce 1000 ml. Standardise the solution immediately before use by titrating with it, in an atmosphere of carbon dioxide, 25 ml of *0.1 M ferric ammonium sulphate* acidified with *sulphuric* acid, using *ammonium thiocyanate* solution, added before the end-point, as indicator.

1 ml of *0.1 M ferric ammonium sulphate* is equivalent to 0.01543 g of $TiCl_3$.

Zinc Chloride, 0.1 M: Dissolve 6.6 g of *granulated zinc*, previously washed with *0.1 M hydrochloric acid* and then with *water*, in the minimum amount of *2 M hydrochloric acid* and add sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

To 25.0 ml of the solution add 4 g of *ammonium acetate* and 25 ml of *water*. Add 50 mg of *xylenol orange mixture* and sufficient *hexamine*, and titrate with *0.1 M disodium edetate* until the colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.013630 g of $ZnCl_2$.

Zinc Sulphate, 0.1 M: Dissolve 29 g of *zinc sulphate* in sufficient *water* to produce 1000 ml. Standardise the solution in the following manner.

Take 20.0 ml solution into a 500 ml conical flask, add 5 ml of *2 M acetic acid* and dilute to 200 ml with *water*. Add about 50 mg of *xylenol orange mixture* and *hexamethylenetetramine* until the solution becomes violet-pink. Add 2 g of *hexamethylenetetramine* in excess. Titrate with *0.1 M disodium edetate* until the violet-pink colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.02875 g of $ZnSO_4,7H_2O$.

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5.1. Cleaning of Glassware

The success of a test or assay of the Pharmocopoeia is determined to a very large extent by the state of cleanliness of the apparatus used. Glassware such as beakers, burettes, flasks, pipettes, etc. should be very clean, especially when employed for certain microbiological assays, the pyrogen test and where small volumes of liquid are measured.

For cleaning glassware, one of the most useful agents is hot *nitric acid.* A very effective cleaning fluid for removing organic matter from glass without heating is by treatment with a chromic acid mixture prepared by dissolving 200 g of *sodium dichromate* in about 100 ml of *water,* cooling in an ice-bath and adding slowly to it, with stirring, 1500 ml of *sulphuric acid.* The mixture should be prepared in a hard, borosilicate glass beaker and safety glasses must be worn during the addition of acid. Chromic acid mixture is extremely corrosive and hygroscopic, and should be stored in glass-stoppered bottles in a safe place. Crystalline chromic acid tends to separate from the mixture on standing, and may be removed by decantation. When the mixture acquires a green colour, it should be discarded under continuously flowing water.

Glass treated with the chromic acid mixture should be subjected to prolonged rinsing as glass tends to adsorb the *chromic acid.* It should not be used for cleaning calibrated containers used for optical measurements.

For general cleaning of glassware, synthetic detergent solutions or alkaline cleansing agent such as *trisodium phosphate* may be used but these also requires prolonged rinsing.

All glassware should be finally rinsed with *purified water* before use.

5.2. Biological Indicators

1. General Requirements

A biological indicator is a microbiological test system consisting of a standardised, viable population of specific microorganisms (usually bacterial spores) inoculated on a carrier contained within its primary pack ready for use and providing a defined resistance to a specified sterilisation process.

Biological indicators provide means to directly assess the microbial lethality of a sterilisation process. When used in conjunction with physical and/or chemical process monitors, biological indicators provide an indication of the effectiveness of a given sterilisation process. A sterilisation process should be considered as satisfactory only when the desired microbiological results, as determined by an appropriate sterilisation cycle development, validation and routine monitoring programme, have been realised. Failure to achieve acceptable microbial challenge results forms the basis for declaring a failure in the sterilisation process. Biological indicators may also be used to evaluate the capability of processes used to decontaminate isolators or aseptic clean-room environments.

2. Types of Biological Indicators

Biological indicators come in various forms designed for specific types of sterilisation. Each type incorporates a viable culture of a known species of microorganisms. There are at least three different types of biological indicators. Some biological indicators may also contain two different species and concentrations of spores.

In one form, the spores are inoculated on a carrier of convenience, such as filter paper placed within a primary pack that protects the carrier from damage or contamination while allowing the sterilant to contact the test organisms. Aseptic technique must be employed when transferring the inoculated carrier to the growth medium in order to avoid accidental contamination. The carrier and primary packaging is not degraded by the specific sterilisation process and is designed to minimise any loss of the original inoculum during transport, handling and shelf-life storage.

In another form, called the self-contained biological indicator, the unit consists of both an ampoule of growth medium and an inoculated carrier with the desired population of test organisms, in another vial. The sterilant enters and exits the outer vial through a filter in the cap. This form of biological indicator together with the self-contained growth medium can be considered a system and the entire system provides resistance to the sterilisation process. To allow for the time lag that may occur while the sterilising agent reaches the contained microorganisms in the system, the *D*-value (Decimal Reduction Value), process end-point, kill time and the survival time should be characterised for the system and not solely for the paper carrier of the self-contained unit. Following processing, the ampoule of growth media is crushed and brought into contact with the inoculated carrier, thereby eliminating the need to aseptically transfer the carrier to a separate vial of growth media.

Other self-contained biological indicators consist of hermetically sealed ampoules containing the test organisms suspended in growth media. The growth medium often also contains a dye, which indicates positive or negative growth following incubation. Due to their construction, this type of biological indicator is sensitive to temperature only and is employed primarily for monitoring sterilisation of liquids. These biological indicators may be incubated directly following exposure to the sterilisation process under the specified conditions. Growth or no growth of the treated spores is

determined visually (either by observing a specified colour change of an indicator incorporated in the medium or by turbidity) or by microscopic examination of the inoculated medium.

The design of the self-contained system is such that there is minimal loss of the original inoculum during transport and handling and the resistance characteristics comply with the labelling of the self-contained system. During or after the sterilisation process, the materials used in the self-contained system do not retain or release any substance that can inhibit the growth of low numbers of surviving indicator microorganism under culture conditions. Adequate steps must be taken to demonstrate that the recovery medium has retained its growth support characteristics after exposure to the sterilisation process.

Custom biological indicators for use in sterilisation-in-place (SIP), or wherever necessary, may be prepared by adding an aliquot of spore suspension to representative units of the lot to be sterilised (inoculated product). If it is not practical to add the spores to the actual product units, spores can be added to a simulated product, which has been validated to demonstrate a similar resistance to the actual product. Spore suspensions with a known *D*-value should be used to inoculate the actual or simulated inoculated product. The population, *D*-value (the time required at a temperature T to reduce a specific microbial population by 90 per cent, or by a factor of 10), *Z*-value (the number of degrees of temperature change necessary to change the *D*-value by a factor of 10), where applicable, and end-point kill time of the inoculated actual or simulated product should be determined.

3. Test Organisms

Test organisms are of a defined strain maintained with a recognised culture collection and identified by appropriate tests.

The preparation of stock spore suspensions of selected microorganisms requires the development of appropriate procedures, including mass culturing, harvesting, cleaning, and maintenance. The stock suspension contains dormant spores that have been held in a non-nutritive liquid. The originating inoculum for each batch of test organism suspension is traceable to the reference culture maintained at a recognised culture collection. The stock suspension is verified as to its identity and purity. Verification tests are specific for each strain of test organism and should be documented by the manufacturer.

4. Labelling

Each batch of biological indicator is accompanied by the following information:

1) name of the biological indicator and intended sterilisation process;
2) a unique code by which the manufacturing history can be traced;
3) the manufacturer's name, trademark, address or other means of identification;
4) the name or abbreviation of the culture collection from which the test organism has been derived and the reference number of the species;
5) the recommended storage conditions, such as, preserving in the original package under the conditions recommended on the label and protecting from light, toxic substances, excessive heat and moisture;
6) the expiry date that is not less than 18 months from the date of manufacture, the date of manufacture being the date on which the first determination of the total viable spore count was made;
7) the total viable spore count of test organisms per unit;
8) the directions for use including incubation conditions, instructions for spore recovery and for safe disposal of the indicator;
9) *D*-value and the method used to determine such *D*-value, i.e., by survivor curve or fraction negative procedure after graded exposures to the sterilisation conditions.
10) indication that the stated *D*-value is reproducible only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result, and that the user should determine the suitability of the biological indicator for the particular use;
11) the survival time and kill time under the specified sterilisation conditions stated on the label.

5. Selection for Specific Sterilisation Processes

The selection of a biological indicator is critical and requires that due weightage be given to the resistance of the biological indicator, to the specific sterilisation process and the material being sterilised so that when used within its performance characteristics, it provides a challenge to the sterilisation process that exceeds the challenge of the natural microbial burden in or on that product.

The effective use of biological indicators for the monitoring of a sterilisation process requires a thorough knowledge of the product being sterilised and its component parts (materials and packaging). It also requires at least a general idea of the probable types and numbers of microorganisms constituting the microbial burden in the product immediately prior to sterilisation.

Only the biological indicator specified for that particular sterilisation process should be used. This will ensure that the biological indicator selected provides a greater challenge to the sterilisation process than the bioburden in or on the product.

When a bioburden-based sterilisation process design is used, data comparing the resistance of the biological indicator to that of bioburden are essential. Enumeration of the bioburden content of the articles being sterilised is also required. The process must result in a biologically verified lethality sufficient to achieve a probability of obtaining a nonsterile unit that is less than one in a million.

Alternatively, the overkill method may be used in the design of a sterilisation process. In this case, specific assumptions are made regarding the resistance used in establishing sterilisation process lethality requirements. In general, all overkill processes are built upon the assumption that the bioburden is equal to one million organisms and that the organisms are highly resistant. Thus, to achieve the required probability of a nonsterile unit that is one in a million, a minimum 12*D* process is required. A 12*D* process is defined as a process that provides lethality sufficient to result in a 12-log reduction, which is equivalent to slightly greater than 12 times a *D*-value for organism with sufficiently higher resistance than the mean resistance of bioburden. Because the bioburden is assumed to be one million, an overkill process will result in a probability of nonsterility at much less than 10^{-6} in actual practice. Overkill process design and evaluation may differ depending upon the sterilisation process under test. The use of an overkill design and validation approach may minimise or reduce the need for bioburden enumeration and identification.

6. Dry Heat Sterilisation Processes

6.1. Test organism. Spores of *Bacillus atrophaeus (Bacillus subtilis* var. *niger)* (ATCC 9372; NCIMB 8058; CIP 77.18) are used for validation and routine monitoring of the performance of sterilisers and sterilising processes employing dry heat at sterilising temperatures, typically at 160°. For routine monitoring, the *D*-value shall not be less than 2.5 minutes.

6.2. Identification. The biological indicator organism complies substantially with the morphological, cultural and biochemical characteristics of the strain of *Bacillus atrophaeus* ATCC 9372; under microscopic examination it consists of Gram-positive rods of width 0.7 µm to 0.8 µm, and length 2 µm to 3 µm, the endospores are oval and central and the cells are not swollen; when incubated aerobically in appropriate media at 30° to 35° growth occurs within 24 hours and similar inoculated media incubated concomitantly at 55° to 60° show no evidence of growth in the same period; agar colonies have a dull appearance and may be cream to brown to orange–coloured; when incubated in nutrient broth it develops a pellicle, and shows little or no turbidity.

6.3. Biological Indicator. The indicator used is Biological Indicator for dry-heat Sterilisation, paper carrier. It is a defined preparation of viable spores made from a culture derived from a specified strain of *Bacillus atrophaeus*, on a suitable grade of paper carrier, individually packaged in a container readily penetrable by dry heat, and characterised for predictable resistance to dry-heat sterilisation. It has a particular labelled spore count per carrier of not less than 10^4 and not more than 10^8.

Dry heat at temperatures greater than 220° is frequently used for Sterilization and depyrogenation of glassware and glass containers. In this case, demonstration of a 3-log reduction in heat resistant bacterial endotoxin can be used as a replacement for biological indicators.

7. Ethylene Oxide Sterilisation Processes

7.1. Test organism. Spores of *Bacillus atrophaeus* (ATCC 9372; NCIMB 8058; CIP 77.18) are used for validating and assessing the routine performance of sterilisers and sterilising processes employing *ethylene oxide*, either as pure ethylene oxide gas or as a mixture of this gas with diluent gases at sterilising temperatures within the range of 30° to 65° and 50 to 70 per cent relative humidity. For routine monitoring, the *D*-value shall be not less than 2.5 minutes at 54° ± 1°; 60 per cent ± 10 per cent RH and 600 mg ± 30 mg /l EO

7.2. Identification. The biological indicator organism complies substantially with the morphological, cultural and biochemical characteristics of the strain of *Bacillus atrophaeus*, ATCC No. 9372, described under Dry heat sterilisation processes.

7.3. Biological indicators. Either of the following may be used.

7.3.1. Biological Indicator for Ethylene Oxide Sterilisation, Paper Carrier. It is a defined preparation of viable spores made from a culture derived from a specified stain of *Bacillus atrophaeus* on a suitable grade of paper carrier, individually packaged in a suitable container readily penetrable by ethylene oxide sterilizing gas mixture and characterized for predictable resistance to sterilization with such gas mixture. It has a particular labelled spore count per carrier of not less than 10^4 and not more than 10^8 spores.

7.3.2. Biological Indicator for Ethylene Oxide Sterilisation, Self-contained. It consists of a Biological indicator for ethylene oxide sterilisation, paper carrier in a suitable container readily penetrable by ethylene oxide sterilising gas mixture and designed to hold an appropriate bacteriological culture medium in a separate ampoule inside the container. After subjection to ethylene oxide sterilisation conditions, the ampoule of growth medium is suitably crushed so as to enable the paper carrier to be immersed and incubated in the growth medium which contains a suitable indicator for determining by a colour change whether or not spores have survived.

8. Moist Heat Sterilisation Processes

8.1. Test organism. Spores of suitable strains of *Geobacillus stearothermophilus (Bacillus stearothermophilus)* (ATCC 7953 & 12980; NCTC 10003; NCIMB 8157; CIP 52.81) are used for validating and assessing routine performance of sterilisers

and sterilising processes employing moist heat as the sterilising agent at temperatures of 121°. For routine monitoring, the D_{121} value shall be not less than 1.5 minutes.

For processes where the temperature employed is less than 121° (sterilisation of heat- sensitive liquids or containers etc.,) microorganisms such as *Bacillus subtilis "5230"* may be used.

8.2. Identification. The biological indicator organism complies substantially with the morphological, cultural and biochemical characteristics of the strain of *Geobacillus stearothermophilus* that is stated in the labelling: under microscopic examination it consists of Gram-positive rods with oval endospores in subterminally swollen cells. When incubated in nutrient broth for 17 hours and used to inoculate appropriate solid media, growth occurs when the inoculated media are incubated aerobically for 24 hours at 55° to 60°, and similar inoculated media incubated concomitantly at 30° to 35° show no evidence of growth in the same period.

8.3. Biological Indicators. Any of the following may be used.

8.3.1. Biological Indicator for Steam Sterilisation, Paper Carrier

It is a defined preparation of viable spores made from a culture derived from a specified strain of *Geobacillus stearothermophilus* on a suitable grade of paper carrier, individually packed in a suitable container readily penetrable by steam and characterised for predictable resistance to steam sterilisation. It has a particular labelled spore count per carrier of not less than 10^4 and not more than 10^8 spores.

8.3.2. Biological Indicator for Steam Sterilisation, Self-contained

It consists of a Biological Indicator for Steam Sterilsation, Paper Carrier individually packaged in a suitable container readily penetrable by steam and designed to hold an appropriate bacteriological culture medium in a separate ampoule. After subjection to saturated steam sterilisation conditions, the ampoule of growth medium is suitably crushed so as to enable the paper carrier to be immersed and incubated in the growth medium which contains a suitable indicator for determining by a colour change whether or not spores have survived.

8.3.3. Biological Indicator for Steam Sterilisation, Sealed Ampoule

It is a defined preparation of viable spores made from a culture derived from a specified strain of *Geobacillus stearothermophilus* which is inoculated into an appropriate bacteriological growth medium, and hermetically sealed in a suitable container. It is sensitive to temperature only and primarily used for steam sterilisation of liquids. After subjection to saturated steam sterilisation conditions, the ampoule is incubated at the specified temperature. The growth medium contains a suitable indicator for determining by a colour change whether or not spores have survived.

9. Ionizing Radiation Sterilising Processes

Spores of *Bacillus pumilus* (ATCC 27142) or other strains or microorganisms of demonstrated equivalent performance are used for validating and assessing routine performance of sterilisers and sterilising processes employing ionising radiation in the form of gamma radiation from a suitable radioisotope source such as Cobalt-60 or Cesium-137 or of electrons energised by a suitable electron accelerator, as the sterilising agent. It has a labelled spore count of not less that 1.0×10^7 and the *D*-value shall be in the range of 0.15 to 0.20 Mrad (1.5 to 2.0 kGy).

For gamma radiation sterilisation, an effective sterilising dose of about 25 kGy (2.5 Mrads) is considered a safe overkill approach. If doses less than 25 kGy are used, additional microbiological monitoring of the product before irradiation will be necessary. In order to validate the efficacy, particularly

Some characteristics of commercially available biological indicators

Sterilisation Mode	Example of a typical *D*-value (minutes)	Minimum *D* values for selecting a suitable biological indicator (minutes)	Minimum survival time (minutes)	Kill time (minutes)
Dry Heat 160°	1.9	Min 1.0 Max 3.0	Min 4.0 Max 14.0	Min 10.0 Max 12.0
Ethylene oxide 600 mg per litre 54°, 60 per cent relative humidity	3.5	Min 2.5 Max 5.8	Min 10.0 Max 27.0	Min25.0 Max 68.0
Moist Heat 121°	1.9	Min 1.5 Max 3.0	Min 4.5 Max 14.0	Min 13.5 Max 32.0

of the lower exposure levels, it is necessary to determine the magnitude (number and/or degree) of the natural radiation resistance of the microbial population of the product.

Radiation dose-setting methods that do not use biological indicators have also been widely used to establish radiation sterilisation processes and effective measurement of the delivered dose can be monitored by physical dosimeters.

10. Vapour-phase Hydrogen Peroxide (VPHP) Sterilising Processes

Spores of *Geobacillus stearothermophilus* are used for validating and assessing the routine performance of sterilisers and sterilising processes employing VPHP. Other microorganisms that may be useful as biological indicators in VPHP processes are spores of *Bacillus atrophaeus* and *Clostridium sporogenes*. Other microorganisms may be considered if their performance responses to VPHP are similar to those of the microorganisms cited above.

The spores may be inoculated on the surface of various carrier systems composed of glass, metal, or plastic surfaces. Highly absorbent surfaces, such as fibrous substrates, or any other substrates that readily absorb VPHP or moisture may adversely influence the VPHP concentration available for inactivation of inoculated microorganism. Paper substrates are not used because VPHP will degrade cellulose-based materials.

The biological indicator may also be individually packaged in a suitable primary overwrap package that does not adversely affect the performance of the indicator, and is penetrable by VPHP. The overwrap materials may facilitate laboratory handling of the biological indicators following exposure to VPHP and must be carefully assessed to ensure that following VPHP exposure, residual hydrogen peroxide is not retained by the packaging materials possibly inducing bacteriostasis during the recovery steps. In cases where biological indicators (inoculated carriers) are being used without the primary package, stringent adherence to aseptic techniques is required.

11. Performance Evaluation of Biological Indicators

11.1. General. When biological indicators are purchased from a commercial source, their suitability for use in a specific sterilisation process should be established through developmental sterilisation studies unless existing data are available to support their use in the process.

Evaluation of the biological indicator must be performed at the time of each validation exercise or with a new lot number or with a change in vendor/manufacturer of biological indicators. The user should establish in-house acceptance standards for biological indicator lots and consider rejection in the event the biological indicator lot does not meet the established in-house performance standards.

The manufacturer's Certificate of Analysis relative to *D*-value range, storage conditions, expiration date, and stability of the biological indicator should be observed and noted. If certificates are not obtained and audits have not been performed, or if the biological indicators are to be used outside of the manufacturer's label claims, verification and documentation of performance under conditions of use must exist.

Upon initial receipt of the biological indicator from a commercial supplier, the user should verify the morphology of the purchased biological indicator microorganism. A microbial count to determine the mean count per biological indicator unit should be conducted.

11.2. Purity. By examination of the spores on a suitable plate culture medium, there should be no evidence of contamination with other microorganisms that will adversely affect the performance of the indicator organism.

11.3. Resistance performance tests. Proceed as given in (14).

11.4. Medium Suitability in Self-contained Biological Indicators

11.4.1. Sterility – Incubate 10 self-contained biological indicator systems at the optimal recovery temperature specified by the manufacturer for 48 hours, making sure that there is no contact between the individual inoculated spore paper carrier and the supplied growth medium. Examine the incubated medium visually (for change in color indicator or turbidity) and microscopically (for absence of microbial growth).

11.4.2. Growth promotion of medium prior to sterilisation treatment – Submerge 10 self-contained units in a water bath maintained at 95° to 100° for 15 minutes. Cool rapidly in an ice water-bath at 0° to 4°. Remove the units from the ice water-bath, immerse the paper carrier in the self-contained medium and incubate at the optimal temperature specified by the manufacturer. Examine visually after 48 hours. All the specimens under test show growth. If one or more specimens do not show growth, repeat the test with 20 additional units.

11.4.3. Ability of medium to support growth after exposure to the sterilisation conditions – Take 10 units after they have been exposed for a kill time as stated in the Certificate of Analysis. Aseptically remove and pool the medium from each unit. Prepare a suspension of the indicator microorganism. Prepare a dilution of that suspension and inoculate the pooled medium with enough suspension to contain a total of 100 to 1000 microorganisms in a 10 ml aliquot obtained from the pooled volume. Incubate the inoculated pooled medium at the optimal temperature specified by the manufacturer. Clear evidence of growth is obtained within 7 days.

12. Use for Validation of Sterilisation Processes

Regardless of the mode of sterilisation, the amount of the initial population of the microorganisms, its resistance to sterilisation and the site of inoculation on or in the product can all influence the rate of biological indicator inactivation.

During microbial challenge, biological indicators should be placed in different pre-identified locations of the product/ materials to be sterilised. If, for example, a container with a closed system is sterilised, both the product solution and the closure should be challenged to ensure that sterilisation equivalent to a 10^{-6} (one in a million probability of a non sterile unit) sterilisation assurance level (SAL) will be obtained in the solution as well as at the closure site. Depending on the locations of the product components most difficult to sterilise, different process parameters may be involved in assuring microbial inactivation to an SAL of 10^{-6}.

The validation phase should identify the most important process parameters for inactivation of microorganisms at the sites most difficult to sterilise. Once these critical processing parameters are determined during validation of the product, they should be operated at the lowest acceptable conditions stated in the sterilisation process specifications.

Considerations leading to different intervals for validation of the sterilisation process could include seasonal changes, product and material changes as well as equipment changes, etc.

13. Use for Routine Monitoring of Sterilisation Processes

13.1. Procedure. Place the biological indicators in the locations determined during the validation process for specific product loads.

After completion of the sterilisation procedure for Biological Indicator for dry_heat sterilisation, paper carrier; Biological Indicator for ethylene oxide sterilisation, paper carrier and Biological indicator for steam sterilisation, paper carrier, whichever is applicable, aseptically remove each paper carrier and add to a sufficient volume of *soyabean-casein digest medium* to submerge the biological indicator in a suitable tube.

For biological indicator for ethylene oxide sterilisation, self-contained and biological indicator for steam sterilisation, self-contained: Crush the self-contained system suitably according to manufacturer's instructions, so as to enable the paper carrier to submerge completely in the growth medium.

Incubate at a temperature of 30° to 35° for biological indicator for dry heat sterilisation and biological indicator for ethylene oxide sterilisation, or at 55° to 60° for biological indicator for steam sterilisation, or in each case at the optimal recovery temperature specified by the manufacturer.

Observe each inoculated medium containing tube, self-contained or sealed ampoule at 24 hours and 48 hours, and every 1 or 2 days thereafter for a total of 7 days of incubation or for the time specified by the manufacturer for products that yield results in less than 7 days. Regardless of self-contained biological indicator or not, growth/no growth should be interpreted according to manufacturers' instructions. Where growth is observed at any particular observation time, further incubation of the specimen(s) concerned is not necessary. Note the specimens showing no evidence of growth at any time.

13.2. Results. A sterilisation process in which all the validated pre-set parameters have been met should show no growth of the biological indicator.

A sterilisation process where validated pre-set minimum parameters have not been met could show growth of the biological indicator.

A sterilisation process where only some of the process parameters have been met might or might not show growth of the indicator.

Actions to be taken upon growth of a biological indicator subsequent to sterilising processing may vary with institutional and regulatory policies, and may require that the lot of the product be rejected as non-sterile. The identification of growth as that of the test organism should be confirmed and an effort made to identify the cause of growth. Consistent growth of biological indicators subsequent to sterilization may indicate a loss of integrity of the sterilisation process or an unusually high resistance of the biological indicator lot under use. If an investigation of the biological indicator indicates no significant change in the biological indicator that affects its performance in the sterilisation process, then the sterilisation process should be requalified.

Any biological indicator test results that show growth of the indicator when no growth would be expected may be an indication of an invalid process, a defective biological indicator or a faulty test system and should lead to an investigation. Unsuitable systems shall be requalified. The identity of the indicator organism should be confirmed for positive biological indicators. If the identity is confirmed, the result should be interpreted as an incomplete sterilisation of the product lot, unless investigation of the biological indicator failure can prove otherwise.

Cultures showing growth that is not confirmed to be the indicator organism should be further investigated to determine the cause of the positive result. Frequent test contaminants may indicate a faulty test system or inadequate training of personnel.

Systems should be established to detect any untoward deviations. This can be achieved by establishing routines for growth promotion tests of the medium or introducing positive controls and tests, as well as, for example, establishing routines for controlling the temperature of the incubator.

The process should be considered acceptable only when the desired physical and/or chemical parameters have been reviewed and the microbiological results interpreted and both found to comply with the desired criteria.

13.3. Disposal. Prior to destruction or discard, sterilise it by steam at 121° for not less than 30 minutes, or by not less than an equivalent method recommended by the manufacturer. This includes a paper carrier used in any test procedures or the paper carriers themselves.

14. Resistance Performance Tests

14.1. Total viable spore count. This test is used to determine the number of viable test organisms both in suspensions and in paper carriers by counting colony forming units (CFUs). It applies to both unprocessed (initial viable count) and processed (viable organisms when using the survivor curve method for *D*-value determination) test samples.

A minimum of four test samples from each lot/batch or exposure shall be used. A homogeneous suspension may be achieved using the following method (glass bead method):

Remove the paper carrier from its original packing and place it in a sterile flat-bottomed, screw-capped 16 x 125 mm test-tube containing three 6-mm glass beads. Add 5.0 ml of sterilised *purified water* and pulp the paper into its component fibres by vortexing vigorously to achieve a homogeneous suspension. Add an additional 5.0 ml of sterilised *purified water.*

Transfer 1.0 ml of the suspension using a pipette with a bore size of at least 2 mm, to a sterile screw-capped 16 x 125 mm test-tube containing 9.0 ml of sterile *purified water*. For biological indicator for steam sterilisation, heat the tube containing the homogeneous suspension in a water- or glycol-bath at 95° to 100° for 15 ± 1 minutes. Start the timing when the temperature reaches 95°. For biological indicator for ethylene oxide sterilsation or biological indicator dry heat sterilisation, heat the tube containing the homogeneous suspension in a water- or glycol-bath at 80° to 85° for 10 minutes. Start the timing when the temperature reaches 80°. Cool rapidly in an ice water-bath at 0° to 4°.

Transfer two 1 ml aliquots of the suspension using a pipette with a bore size of at least 2 mm to duplicate tubes containing 9.0 ml of sterile *purified water*. Make appropriate serial dilutions in sterile *purified water*, the dilutions being adjusted to yield between 30 and 300 colonies, on each of a pair of plates when treated as described below. Where the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution.

Prepare a separate series of plates for each aliquot. Place 1.0 ml of each selected dilution in each of two petri dishes 9 to 10 cm in diameter. Add to each plate 20 ml of casein- soyabean digest agar medium that has been melted and cooled to 55°. Swirl to attain a homogeneous suspension and allow to solidify. Incubate the plates in an inverted position at 55° to 60° for biological indicator for steam sterilisation and at 30° to 35° for biological indicator for ethylene oxide sterilisation or biological indicator for dry heat sterilisation, or at the optimal recovery temperature specified by the manufacturer of the biological indicator. Incubate for 48 to 72 hours. Units incubated at 55° to 60° may be placed inside a plastic bag to prevent the plates from excessive drying.

Examine the plates after 24 and 48 hours, recording for each plate the number of colonies, and using the number of colonies after 48 hours to calculate the results. Calculate the average number of spores per sample from the results, using the appropriate dilution factor.

For biological indicators, sealed ampoule, take 4 ampoules in a sterile 125 ml screw-capped glass bottle and break the ampoules by shaking against the sides of the bottle. Add sufficient sterile *purified water* to make 100.0 ml and vortex or sonicate vigorously to achieve a homogeneous suspension. Transfer 10.0 ml to a sterile 16 x 125 mm screw-capped tube. Carry out the procedure described above beginning at the words " Transfer 1.0 ml of the suspension.....".

The requirements of the test are met if the log average number of the viable spores per carrier is not less than 0.3 log and does not exceed the log labelled spore count per carrier by 0.48.

14.2. Determination of *D*-Value. Users of biological indicators are generally not required to perform resistance assays but they may have differing requirements for their quality assurance systems. The requirement to perform *D*-value confirmation testing can be eliminated by an appropriate audit of the manufacturer of the biological indicator. This can be accomplished in one of three ways viz. a personal audit by the user of the facilities of the manufacturer of the indicator, performance of a "mail-in" audit, or confirmation by the indicator manufacturer that an audit has been performed by a notified body (e.g., certification of comparison to standards ISO 10011-1, 10012-1 and 10011-3).

A Certificate of Analysis that a biological indicator conforms to a specific resistance characteristic is required. If certificates are not obtained and audits have not been performed, or if the biological indicators are to be used outside of the manufacturer's label claims, verification and documentation of resistance performance, under conditions of use, must be confirmed.

The user may consider conducting a *D*-value assessment before acceptance of the lot. Laboratories that have the capability of performing *D*-value assays may determine the *D*-

value using the method cited in ISO document *ISO 11138-1:1994(E) Annexes C and/or D*. If the user elects to reconfirm the *D*-value specified on the Certificate of Analysis, testing must be by the method used by the manufacturer.

14.3. Survival time and Kill time. When labelled for and subjected to a specified sterilisation, a biological indicator has a survival time and kill time appropriate to the labelled spore count and to the decimal reduction value (*D*-value in minutes) of the indicator, specified as:

Survival time (in minutes) = not less than (labelled *D*-value x (log labelled spore count per carrier – 2);

Kill time (in minutes) = not more than (labelled *D*-value x (log labelled spore count per carrier + 4).

14.3.1. Procedure. Take four groups, each consisting of 25 specimens of the relevant biological indicator, in their original individual packages. Place the specimens of each group in suitable specimen holders that permit each specimen to be exposed to the sterilising conditions at a specific location in the sterilising chamber. Check the chamber for operating parameters by preheating it to the selected temperature ± 1° in the cases of biological indicator for dry heat sterilisation, and biological indicator for ethylene oxide sterilisation or ±0.5° in the case of biological indicator for steam sterilisation.

For biological indicator for dry heat sterilisation, preheat the unit to temperature, and equilibrate the heat chamber. Open the access door or port, and place the holder(s) in the chamber, close the access door or port, and continue to operate the apparatus. Commence timing the heat exposure when the chamber temperature returns to the lower limit of the selected temperature. Expose the first group of 25 specimens for the required survival time, enter the chamber, and remove the holder(s) containing the specimens. Repeat this test with the second group of 25 specimens. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, with the last two groups, each consisting of 25 specimens, similarly, but expose the specimens for the required kill time.

For biological indicator for ethylene oxide sterilisation, preheat the chamber to equilibrium at the selected temperature 54 ± 1°, and proceed as follows:

1. Evacuate the test chamber to a pressure of not more than 100 ± 3 mm of mercury.
2. Inject sufficient water vapor (e.g. saturated steam) to bring the chamber contents to within 10 per cent relative humidity of the required humidification condition, and allow the chamber to equilibrate with moisture and to temperature for about 30 minutes.
3. Inject a sufficient quantity of temperature-equilibrated ethylene oxide gas to attain the appropriate concentration of ± 30 mg of ethylene oxide per litre.
4. Subject a group of specimens to the appropriate temperature, humidification, and gas concentration conditions for the required survival time.
5. Evacuate the test chamber to a pressure of 100 ± 3 mm of mercury, and release the vacuum with sterile filtered air. Repeat this until not less than 99 per cent of the remaining gas has been removed, and remove the holder(s) with the exposed specimens.

Repeat the above procedure with the second group of 25 specimens and the last two groups each consisting of 25 specimens, similarly, but using a gassing time in step (4) appropriate to the required kill time.

For biological indicator for steam sterilisation, paper carrier and biological indicator for steam sterilisation, sealed ampoule, exhaust the steam chamber and open the door. Within 15 seconds of opening the door place the loaded holder(s) into the chamber, and operate the apparatus to heat the chamber contents to 121° in less than 10 seconds. Expose the specimens for the required survival time, counting the exposure time from when the temperature record shows that the chamber has reached the required temperature. Exhaust the chamber in less than 10 seconds at the end of exposure period. When the chamber can be safely entered, remove the holder(s) containing the specimens. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, so as to subject the holder(s) containing 25 specimens similarly to the first exposure. Repeat the above procedure with two more groups each consisting of 25 specimens, similarly, but expose the specimens for the calculated kill time.

14.3.2. Recovery. After completion of the sterilisation procedure incubate all biological indicators. All self-contained biological indicators should be activated as necessary and incubated in their own medium. For all other biological indicators, aseptically remove each spore carrier and add to a sufficient volume of soyabean-casein digest medium to submerge the carrier in a suitable tube Use an incubation temperature of 30° to 35° in the cases of biological indicator for dry heat sterilisation and biological indicator for ethylene oxide sterilisation, paper carrier, and 55° to 60° in the case of biological indicator for steam sterilisation, or at the optimal temperature specified by the manufacturer.

Observe each inoculated medium tube daily for the length of time specified by the manufacturer. If no time is specified, incubate for 7 days after inoculation. Growth/no growth should be interpreted according to the manufactures' instructions. Where growth is observed at any particular observation time, further incubation of the specimen(s) is not necessary. Note the number of specimens showing no evidence of growth at each observation as well.

The requirements of the test are met if all of the specimens subjected to the specified sterilisation for the survival time

show evidence of growth, while none of the specimens subjected to the specified sterilisation for the kill time shows growth.

If for either the survival time test or the kill time test, not more than one specimen out of both groups fails the survival requirements or the kill requirements (whichever is applicable), repeat the corresponding test with four additional groups each consisting of 20 specimens, according to the procedure described. If all of the additional specimens subjected to the specified process either meet the survival requirement for the survival time or meet the kill requirement for the kill time test, whichever is applicable, the requirements are met.

5.3. Sterilisation

Sterility is the total absence of viable microorganisms. Absolute sterility cannot be practically demonstrated without complete destruction of every finished article. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. The sterility of an entire lot of finished compendial article cannot be guaranteed by testing; it has to be assured by the application of a suitably validated production process. Such a process would involve the use of adequate sterilisation cycles and subsequent aseptic processing, if any, under appropriate good manufacturing practices, and not by reliance solely on sterility testing. The design of the process should include the use of:

- qualified personnel with appropriate training,
- adequate premises,
- suitable production equipment, designed for easy cleaning and sterilisation,
- adequate precautions to minimize the bioburden prior to sterilisation,
- validated procedures for all critical production steps,
- environmental monitoring and in-process testing procedures.

The precautions necessary to minimize the pre-sterilisation bioburden include the use of components with an acceptable degree of microbial contamination. Microbiological monitoring and setting of suitable acceptance or action limits may be required for ingredients that are liable to be contaminated because of their origin, nature or method of preparation.

Failure to follow meticulously a validated process involves the risk of a non-sterile product or even of a deteriorated product.

NOTE — The methods described here apply mainly to the inactivation of bacteria, yeasts and moulds. For biological products of animal or human origin or in cases where such material has been used in the production process, it is necessary during validation to demonstrate that the process is capable of removing or inactivating relevant viral contamination.

The basic steps of validation of a sterilizing process consist of:

1. Qualifying the process equipment in order to demonstrate that it has the capability of operating within the required parameters.
2. Demonstrating that the critical control equipment and instrumentation are capable of operating within the prescribed parameters for the process equipment.
3. Performing replicate cycles representing the required operational range of the equipment and employing actual or simulated product and demonstrating that the processes have been carried out within the prescribed protocol limits and that the probability of microbial survival in the replicate processes completed is not greater than the prescribed limits.
4. Monitoring the validated process during routine operation.
5. Requalifying and rectifying the equipment periodically, as needed.
6. Completing the protocols, and documenting the above-stated steps.

Proper validation of the sterilisation process or the aseptic process requires a high level of knowledge of the field of sterilisation and clean room technology. To meet the currently acceptable and achievable limits in sterilisation parameters, it is essential to employ appropriate instrumentation and equipment to control the critical parameters such as temperature and time, humidity, and sterilising gas concentration, or absorbed radiation.

Given below is a typical multi-stage validation programme designed for steam sterilisation, but the same principles apply to other sterilisation procedures. A vital aspect of the validation programme in many sterilisation procedures is the use of biological indicators (see 5.2. Biological Indicators).

Validation begins with the qualification of the sterilising equipment. The installation qualification stage is intended to establish that controls and other instrumentation are properly designed and calibrated. There should be documented evidence of the quality of the required utilities such as water, steam, and air. The operational qualification stage is intended to confirm that the empty chamber functions within the parameters of temperature at all key positions of the chamber prescribed in the validation protocol. Heat profile records using multiple temperature-sensing devices and indicating simultaneous temperatures in the chamber are created. A typical

acceptable range of temperature in the empty chamber is ± 1° when the chamber temperature is not less than 121°. The performance qualification stage of the validation programme is the actual sterilisation of materials or articles. This determination requires the use of temperature-sensing devices inserted into samples of the articles as well as either samples of the articles to which appropriate concentrations of suitable test microorganisms have been added, or separate biological indicators in operationally fully-loaded autoclave configurations. The effectiveness of heat delivery or penetration into the actual articles and the time of exposure are the two main factors that determine lethality of the sterilization process. The final stage of the validation programme requires the documentation of the data developed in executing the programme.

The validated process should be revalidated periodically; however, the revalidation programme need not be as extensive as the original one. Revalidation is also required whenever major changes in the sterilisation procedure, including changes in the load, take place.

1. Sterility Assurance Level (SAL)

The achievement of sterility within any one item in a population of items submitted to a sterilisation process cannot be guaranteed nor can it be demonstrated. The inactivation of microorganisms by physical or chemical means follows an exponential law; thus, there is always a finite statistical probability that a microorganism may survive the sterilising process. For a given process, the probability of survival is determined by the number, types and resistance of the microorganisms present and by the environment in which the organisms exist during treatment. The sterility assurance level (SAL) of a sterilising process is the degree of assurance with which the process in question renders a population of items sterile. The SAL for a given process is expressed as the probability of a non-sterile item in that population. An SAL of 10^{-6}, for example, denotes a probability of not more than one viable microorganism in $1x10^6$ sterilised items of the final product. The SAL of a process for a given product is determined by appropriate validation studies.

2. Sterilisation

Wherever possible, a process in which the product is sterilised in its final container (terminal sterilisation) is chosen. If terminal sterilisation is not possible, filtration through a bacteria-retentive filter and aseptic processing should be used; wherever possible, appropriate additional treatment of the product (e.g. heating of the product) in its final container may be applied. In all cases, the container and closure are required to maintain the sterility of the product throughout its shelf-life.

It is generally accepted that terminally sterilised injectable articles attain an SAL level of 10^{-6}. With heat-stable articles, the approach often is to considerably exceed the critical time necessary to achieve the 10^{-6} microbial survival probability (overkill). However, with an article where excessive heat exposure is likely to damage the product, it may not be feasible to employ this overkill approach. In such cases, the development of the sterilisation cycle depends heavily on knowledge of the microbial burden of the product. Where the *D*-value [the time in minutes required to reduce the microbial population by 90 per cent or 1 log cycle (i.e., to a surviving fraction of 1/10), at a specific temperature] of a biological indicator preparation of, for example *Bacillus stearothermophilus* spores is 1.5 minutes under the total process parameters, e.g., at 121°, if it is treated for 12 minutes under the same conditions, it can be stated that the lethality input is 8*D*. The effect of applying this input to the product would depend on the initial microbial burden. Assuming that its resistance to sterilisation is equivalent to that of the biological indicator, if the microbial burden of the product in question is 10^2 microorganisms, a lethality input of 2*D* yields a microbial burden of 1 (10^0 theoretical) and a further 6*D* yields a calculated microbial survivor probability of 10^{-6}. Under the same conditions, a lethality input of 12*D* may be used in a typical "overkill" approach. For valid use, it is essential that the resistance of the biological indicator be greater than that of the natural microbial burden of the article sterilised. In the above example, a 12-minute cycle is considered adequate for sterilisation if the product had a microbial burden of 10^2 microorganisms. However, if the indicator originally had 10^6 microorganisms content, actually a 10^{-2} probability of survival could be expected; i.e. 1 in 100 biological indicators may yield positive results. This type of situation may be avoided by selection of the appropriate biological indicator. Alternatively, high content indicators may be used on the basis of a predetermined acceptable count reduction.

Any microbial burden method for sterility assurance requires adequate surveillance of the microbial resistance of the article to detect any changes, in addition to periodic surveillance of other attributes.

3. Methods of Sterilisation

Sterilisation may be carried out by one of the methods described below. The choice of the appropriate method for an article requires a high level of knowledge of sterilisation techniques and information regarding any effects of the sterilising process on the material being sterilised. Modifications to, or combination of, the methods given may be used provided that the chosen procedure is validated both with respect to its effectiveness and the integrity of the product including its container or package.

For all methods of sterilisation the critical conditions of the operation are monitored in order to confirm that the previously determined required conditions are achieved throughout the

batch during the sterilisation process. This applies even in cases where the reference conditions are used.

3.1. Steam sterilisation (Heating in an autoclave). This process of thermal sterilisation using saturated steam under pressure should be used whenever possible for aqueous preparations and for surgical materials. The process is based on the principle of displacing the air in the sterilising chamber (autoclave) by saturated steam and continuing the exposure to saturated steam for a period of time sufficient to ensure that the entire articles being sterilised are maintained for an effective combination of time and temperature to ensure sterility. In order to displace air more effectively from the chamber and from within the articles, the sterilisation cycle may include air and steam evacuation stages. The choice of a cycle for any article depends on a number of factors, including the heat lability of the material, knowledge of heat penetration into the articles, and other factors. The reference conditions for aqueous preparations are heating at a minimum of 121° for 15 minutes. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operating routinely within the established tolerances. The procedures and precautions employed should be such as to give an SAL of 10^{-6} or better.

In establishing the sterilisation cycle parameters the F_0 concept may be used. The F_0 at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time. In general for aqueous preparations a microbiologically-validated steam sterilisation process that delivers, in total (including the heating up and cooling down phases of the sterilising cycle), an F_0 value of not less than 8 to every container in the autoclave load is considered satisfactory. If a product is especially heat-sensitive or in certain circumstances where a process that delivers, in total, an F_0 less than 8 is necessary, great care must be taken to ensure that an SAL of 10^{-6} or better is consistently achieved.

When surgical materials are sterilised the steam used should neither be superheated nor contain more than 5 per cent of entrained moisture. Appropriate steps should be taken to ensure adequate removal of air. Most dressings are conveniently sterilised by maintaining at a temperature of 134° to 138° for 3 minutes but other suitable combinations of temperature and time may be used.

3.2. Dry heat sterilisation. This process of thermal sterilisation is suitable for heat-stable, non-aqueous products and powders. The reference conditions are a minimum of 160° for at least 2 hours; other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate level of lethality when operated routinely within the established tolerances. The procedures and precautions employed should be such as to give an SAL of 10^{-6} or better.

The process of sterilisation is carried out by a batch process in an oven specially designed for that purpose. The oven is supplied with heated, filtered air, distributed uniformly throughout the chamber by convection or radiation employing a blower system with devices for sensing, monitoring, and controlling the critical parameters. The steriliser is loaded in such a way that a uniform temperature is achieved throughout the load. The temperature-sensing elements are inserted into representative containers together with additional elements at the previously established coolest part of the loaded steriliser. An acceptable range in temperature in the empty chamber is ±15° when the unit is operating at not less than 250°. Where the unit is employed for sterilising components such as containers intended for intravenous solutions, care should be taken to avoid accumulation of particulate matter in the chamber.

SAL of 10^{-12} is considered achievable for heat-stable articles or components.

3.3. Gas sterilisation. This method of sterilisation is only to be used when the material to be sterilised cannot withstand the high temperatures obtained in the steam sterilisation or dry heat sterilisation processes. The active agent generally used is *ethylene oxide* of acceptable sterilising quality. The sterilisation process is generally carried out in a pressurised chamber designed similarly to a steam autoclave but with additional features, particularly for ensuring the reduction of gas or its transformation products in the sterilised product to below the concentration that could give rise to toxic effects during the use of the product.

The programme for the validation of the sterilising process is more comprehensive than for the other sterilisisation procedures, since in addition to temperature, the humidity, vacuum/positive pressure, and ethylene oxide concentration also require rigid control. It is important to demonstrate that all critical process parameters in the chamber are adequate during the entire cycle. Since the sterilisation parameters applied to the articles to be sterilised are critical variables, it is advisable to precondition the load to achieve the required moisture content, to minimise the time of holding at the required temperature, prior to placement of the load in the ethylene oxide chamber. The validation is generally done using the product inoculated with appropriate biological indicators.

Wherever possible, the gas concentration, relative humidity, temperature and duration of the process are measured and recorded. Measurements are made where sterilisation conditions are least likely to be achieved, as determined at validation. The effectiveness of the process applied to each sterilisation load is checked using a suitable biological indicator.

One of the principal limitations of the gas sterilisation process is the limited ability of the gas to diffuse to the innermost product areas that require sterilisation. Package design and chamber loading patterns must therefore be determined so the there is minimum resistance to gas diffusion.

3.4. Ionising radiation sterilisation. Sterilisation by this method is achieved by exposure of the product to ionising radiation in the form of gamma radiation from a suitable radioisotopic source (such as cobalt 60 or cesium 137) or of a beam of electrons energised by a suitable electron accelerator.

The advantages of the method include low chemical reactivity, low measurable residues, and the fact that there are fewer variables to control. The unique feature of the method is that the basis of control is essentially that of the absorbed radiation dose, which can be precisely measured. Irradiation causes only a minimal rise of temperature, but can affect certain grades of plastics and glass.

The two types of ionising radiation in use are radioisotope decay (gamma radiation) and electron-beam radiation. In either case, the radiation dose to yield the required degree of sterility assurance should be established such that within the range of minimum and maximum doses set, the properties of the article being sterilised are acceptable.

For gamma radiation, the validation of a procedure should include the establishment of article materials compatibility, establishment of product loading pattern and completion of dose mapping in the sterilisation container, establishment of timer setting, and demonstration of the delivery of the required sterilisation dose. For electron-beam irradiation, in addition, the on-line control of voltage, current, conveyor speed, and electron beam scan dimension must be validated.

For gamma radiation the reference absorbed dose is 25 kGy (2.5 megarads). Other doses may be used provided that it has been satisfactorily demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed should be such as to give an SAL of 10^{-6} or better. In order to validate the efficacy, particularly of the lower exposure levels, it is necessary to determine the magnitude of the natural radiation resistance of the microbial population of the product. Specific product loading patterns must be established and absorbed minimum and maximum dosage distribution must be determined by the use of chemical dosimeters. These dosimeters should be calibrated against a standard source at a reference radiation plant.

The setting of the preferred absorbed dose should be carried out using a suitable biological indicator.

3.5 Filtration. Certain active ingredients and products that cannot be terminally sterilised may be subjected to filtration through microbial retentive materials. The suitability of filters of such materials should have been demonstrated by means of a microbial challenge test using a suitable test microorganism. A suspension of *Pseudomonas diminuta* (ATCC 19146, NCIMB 11091) may be suitable. It is recommended that a challenge of at least 10^7 CFU per cm^2 of active filter surface is used and that the suspension is prepared in *tryptone soya broth* which, after passage through the filter, is collected aseptically and incubated aerobically at 32°. Such filter membranes are nominally rated 0.22 µm or 0.2 µm.

It must be ascertained whether the filtration parameters employed in manufacturing the product will significantly influence microbial retention efficiency. Some of the other factors in validation of the filtration process include product compatibility, absorption of drug, preservative and/or other additives, release of contaminants from the filter and initial effluent endotoxin content.

Since the effectiveness of the filtration process is also influenced by the microbial burden of the solution to be filtered, the determination of the microbiological quality of solutions prior to filtration is also an important aspect of validation of the process in addition to other parameters such as pressures, flow rates, and filter unit characteristics. The production process and environment should be designed to minimise microbial contamination and should be regularly subjected to appropriate monitoring procedures. The equipment, containers, and closures and, where possible, the ingredients should be subjected to an appropriate sterilisation process. It is recommended that the filtration process is carried out as close as possible to the filling point. Attention should be given to the filter capacity, batch size and duration of filtration. The filter should not be used for a longer period than has been approved by validation of the combination of the filter and the product in question. The operations following filtration should be carried out under aseptic conditions.

The integrity of an assembled sterilising filter should be verified before use and confirmed after use by carrying out tests appropriate to the type of filter used and the stage of testing, e.g., bubble-point, pressure hold or diffusion rate tests.

Due to the potential additional risks of the filtration method as compared with other sterilisation processes, a prefiltration through a bacteria-retentive filter (e.g. 0.45 µm pore size) may be necessary in cases where a low bioburden cannot be ensured by other means.

5.4. Residual Solvents

Residual solvents are defined as organic volatile impurities that may remain in active pharmaceutical substances, excipients, or medicinal products after processing. During the manufacturing processes, the solvents are not completely removed. The solvents may be used to improve the yield in the synthesis of active pharmaceutical substances besides

imparting characteristics of crystal form, purity and solubility. The use of solvents may, therefore, have a critical role in the synthetic process. Nevertheless, testing should be done for residual solvents when production or purification processes are known to result in the presence of such solvents. It is only necessary to test for solvents that are used or produced in the manufacture or purification of active substances, excipients or dosage forms.

Residual solvents do not have any therapeutic effect. Therefore, efforts should be made to remove them to the extent possible to meet the specifications prescribed. Tests for residual solvents are not generally mentioned in specific monographs since the solvents employed may vary from one manufacturer to another and the requirements of this general chapter are applied by adopting the Good Manufacturing Practices and/or other quality based requirements. Normally, the competent authority is to be informed of the solvents employed during the production process. Pharmaceutical products should contain no higher levels of residual solvents than can be supported by safety data.

Depending upon the safety data and their risk to the human health, these solvents are classified as follows:

Class 1 solvents — These solvents are known to cause unacceptable toxicities and should be avoided in the manufacture of active pharmaceutical substances, excipients and medicinal products. However, if their use is unavoidable, the restricted concentration limits shown in Table 1 should be applied.

Table 1

Solvent	Concentration Limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon Tetrachloride	4	Toxic and environmental hazard
1, 2-Dichloroethane	5	Toxic
1, 1-Dichloroethene	8	Toxic
1, 1, 1-Trichloroethane	1500	Environmental hazard

Class 2 solvents — These solvents are associated with less severe toxicity but should be limited in pharmaceutical products for the protection of consumers from potential adverse effects. Concentration limits shown in Table 2 are applicable if any solvent of this class is used.

Table 2

Solvent	PDE (mg/day)	Concentration Limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cumene	0.7	70
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
N,N-Dimethylacetamide	10.9	1090
N,N-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol**	1.6	160
Ethyleneglycol**	6.2	620
Formamide**	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol**	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
Methylisobutylketone	45	4500
N-Methylpyrrolidone**	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200
Sulpholane**	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
1, 1, 2-Trichloroethene	0.8	80
Xylene*	21.7	2170

* Usually 60 per cent *m-xylene*, 14 per cent *p-xylene*, 9 per cent, *o-xylene* with 17 per cent ethyl benzene.

** Not readily detected by head-space injection gas chromatography. Other appropriate procedures should be used for control purposes.

Options for describing limits of class 2 solvents—Two options are available when setting limits for Class 2 solvents.

Option 1. The concentration limits in ppm stated in Table 2 can be used. They were calculated using equation (1) below by assuming a product mass of 10 g administered daily.

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{dose}}$$

Here, PDE is given in terms of mg/day and dose is given in g per day.

These limits are considered acceptable for all substances, excipients, or products. Therefore this option may be applied if the daily dose is not known or fixed. If all excipients and drug substances in a formulation meet the limits given in Option 1, then these components may be used in any proportion. No further calculation is necessary provided the daily dose does not exceed 10 g. Products that are administered in doses more than 10 g per day should be considered under Option 2.

Option 2. It is not considered necessary for each component of the drug product to comply with the limits given in Option 1. The PDE in terms of mg per day as stated in Table 2 can be used with the known maximum daily dose and equation (1) above to determine the concentration of residual solvent allowed in a drug product. Such limits are considered acceptable provided that it has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, reasonable variation in the manufacturing process, and the limits should reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the drug product. The sum of the amounts of the solvent per day should be less than that given by the PDE.

Consider an example of the use of Option 1 and Option 2 applied to acetonitrile in a drug product. The permitted daily exposure to acetonitrile is 4.1 mg/day; thus the Option 1 limit is 410 ppm. The maximum administered daily mass of a drug product is 5.0 g , and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Drug substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	400 ppm	0.36 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Drug product	5.0 g	728 ppm	3.64 mg

Excipient 1 meets the Option 1 limit, but the drug substance, excipient 2, and drug product do not meet the Option 1 limit. Nevertheless, the product meets the option 2 limit of 4.1 mg per day and thus conforms to the recommendations in this guideline.

Consider another example using acetonitrile as residual solvent. The maximum administered daily mass of a drug product is 5.0 g, and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile is given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Drug substance	0.3 g	800 ppm	0.2 mg
Excipient	0.9 g	2000 ppm	1.80 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Drug product	5.0 g	1016 ppm	5.08 mg

In this example, the product meets neither the Option 1 nor the Option 2 limit according to this summation. The manufacturer could test the drug product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the drug product should take other steps to reduce the amount of acetonitrile in the drug product. If all of these steps fail to reduce the level of residual solvent, in exceptional cases the manufacturer could provide a summary of efforts made to reduce the solvent level to meet the guideline value, and provide a risk-benefit analysis to support allowing the product to be utilized with residual solvent at a higher level.

Class 3 solvents — These solvents are less toxic and of lower risk to human health. Nevertheless, they need to be limited by good manufacturing practices or other quality-based requirements. The concentration limits of 5000 ppm would be acceptable for the solvents listed in Table 3.

Table 3

Acetic Acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl Acetate	Methylethylketone
tert-Butylmethyl Ether	2-Methyl-1-propanol
Dimethyl Sulphoxide (DMSO)	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	Triethylamine

Test procedures

Chromatographic techniques like gas chromatography are generally suitable for determining levels of residual solvents. A non-specific method like Loss on drying may be used if only Class 3 solvents are present. However, if for a Class 3 solvent a justified limit higher than 0.5 per cent is applied, a specific determination of the solvent is required. The test methods described in this general method may be used:

— to identity the majority of the residual solvents of Class 1 and Class 2 in an active pharmaceutical substance, excipient or medicinal product when the residual solvents are unknown;

— as a limit test for Class 1 and Class 2 solvents when present in an active pharmaceutical substance, excipient or medicinal product;

– to quantify Class 2 solvents (with limits greater than 1000 ppm) and Class 3 solvents when required.

Three diluents for sample preparation and two sets of chromatographic conditions are described for examination by gas chromatography.

Procedure

Solutions

Sample solution (1). For water-soluble substances. Dissolve 0.2 g of the substance under examination in *water* and dilute to 20.0 ml with the same solvent.

Sample solution (2). For water-insoluble substances. Dissolve 0.2 g of the substance under examination in *N,N-dimethylformamide* and dilute to 20.0 ml with the same solvent.

Sample solution (3). For substances known or suspected to contain *N,N-dimethylacetamide* and/or *N,N-dimethylformamide*. Dissolve 0.2 g of the substance under examination in *3-dimethyl-2-imidazolidine* and dilute to 20.0 ml with the same solvent.

NOTE — Where none of the above sample preparation procedures are appropriate, the diluent to be used and the chromatographic conditions to be used must be validated.

Solvent solution (a). To 1.0 ml of Class 1 residual solvent mixture, add 9 ml of *dimethylsulphoxide* and dilute to 100.0 ml with *water*. Dilute 1.0 ml of this solution to 100.0 ml with *water*. Dilute 1.0 ml of the resulting solution to 10.0 ml with *water*.

Solvent solution (b). Dissolve appropriate quantities of the Class 2 residual solvents in *dimethylsulphoxide* and dilute to 100.0 ml with *water*. Dilute to give a concentration of 1/20 of the limits stated in Table 2.

Solvent solution (c). Dissolve 1.0 g of the solvent or solvents present in the substance under examination in *dimethyl sulphoxide* or *water*, if appropriate, and dilute to 100.0 ml with *water*. Dilute to give a concentration of 1/20 of the limits stated in Table 1 or 2.

Blank solution. Prepare as described for solvent solution (c) but without the addition of solvent(s) (for verifying the absence of interfering peaks).

Test solution. Introduce 5.0 ml of the sample solution and 1.0 ml of the blank solution into an injection vial.

Reference solution (a) (Class 1). Introduce 1.0 ml of solvent solution (a) and 5.0 ml of the appropriate diluent into an injection vial.

Reference solution (a1) (Class 1). Introduce 5.0 ml of the sample solution and 1.0 ml of solvent solution (a) into an injection vial.

Reference solution (b) (Class 2). Introduce 1.0 ml of solvent solution (b) and 5.0 ml of the appropriate diluent into an injection vial.

Reference solution (c). Introduce 5.0 ml of the sample solution and 1.0 ml of solvent solution (c) into an injection vial.

Reference solution (d). Introduce 1.0 ml of the blank solution and 5.0 ml of the appropriate diluent into an injection vial.

Close the vials with a light rubber membrane stopper coated with polytetrafluoroethylene and secure with aluminium crimped caps. Shake to obtain homogeneous solutions.

Method

Determine by gas chromatography with static head-space injection (2.4.13).

Injection conditions:

Operating parameters	Sample solution No (1)	(2)	(3)
Equilibration temperature (°)	80	105	80
Equilibration time (minutes)	60	45	45
Transfer-line temperature (°)	85	110	105
Pressurisation time (seconds)	30	30	30
Injection volume (ml)	1	1	1

Chromatographic system 1

- a fused-silica capillary column 30 m × 0.32 mm or 0.53 mm, coated with cross-linked 6 per cent polycyanopropylphenylsiloxane and 94 per cent polydimethylsiloxane (film thickness: 1.8 µm or 3 µm),
- temperature: column 40° for 20 minutes, then raised to 240° at a rate of 10° per minute and maintained at 240° for 20 minutes,
- inlet port at 140° and detector at 250°,
- carrier gas: *Nitrogen for chromatography* at an appropriate pressure flow,
- a flame-ionisation detector (or a mass spectrometer or an electron-capture detector for the chlorinated solvents of Class 1).

If there is interference from the matrix, use the following system:

Chromatographic system 2

- a fused-silica capillary column 30 m x 0.32 mm or 0.53 mm, coated with macrogol 20000 (film thickness: 0.25 µm),
- temperature: column 50° for 20 minutes, then raised to 165° at a rate of 6° per minute and maintained at 165° for 20 minutes,
- inlet port at 140° and detector at 250°,
- carrier gas: *Nitrogen for chromatography* at an appropriate pressure flow,

– a flame-ionisation detector (or a mass spectrometer or an electron-capture detector for the chlorinated solvents of Class 1).

Inject 1 ml of the gaseous phase of reference solution (a) into the column of system 1 and record the chromatogram under such conditions that the signal-to-noise ratio for 1,1,1-trichloroethane can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Fig. 5.4-1.

Inject 1 ml of the gaseous phase of reference solution (a1) into the column of system 1. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 ml of the gaseous phase of reference solution (b) into the column of system 1 and record the chromatogram under such conditions that the resolution between acetonitrile and methylene chloride can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Fig. 5.4-2 and the resolution between acetonitrile and methylene chloride is at least 1.0.

Inject 1 ml of the gaseous phase of the test solution into the column of system 1. If in the chromatogram obtained there is no peak which corresponds to one of the residual solvent peaks in the chromatograms obtained with reference solution (a) or (b), then the substance under examination meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) then system 2 is to be used.

Inject 1 ml of the gaseous phase of reference solution (a) into the column of system 2 and record the chromatogram under such conditions that the signal-to-noise ratio for benzene can be measured. The signal-to-noise ratio is shown in Fig. 5.4-3.

Inject 1 ml of the gaseous phase of reference solution (a1) into the column of system 2. The peaks due to Class 1 residual solvents are still detectable.

Inject 1 ml of the gaseous phase of reference solution (b) into the column of system 2 and record the chromatogram under

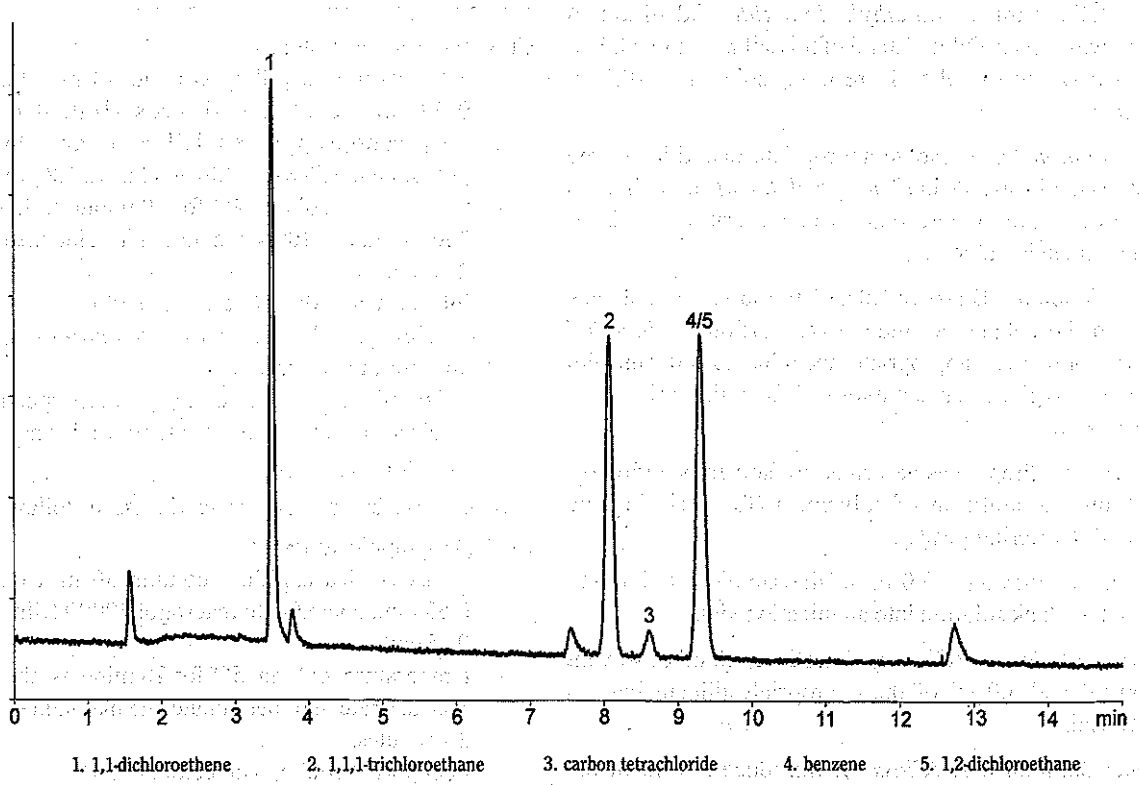
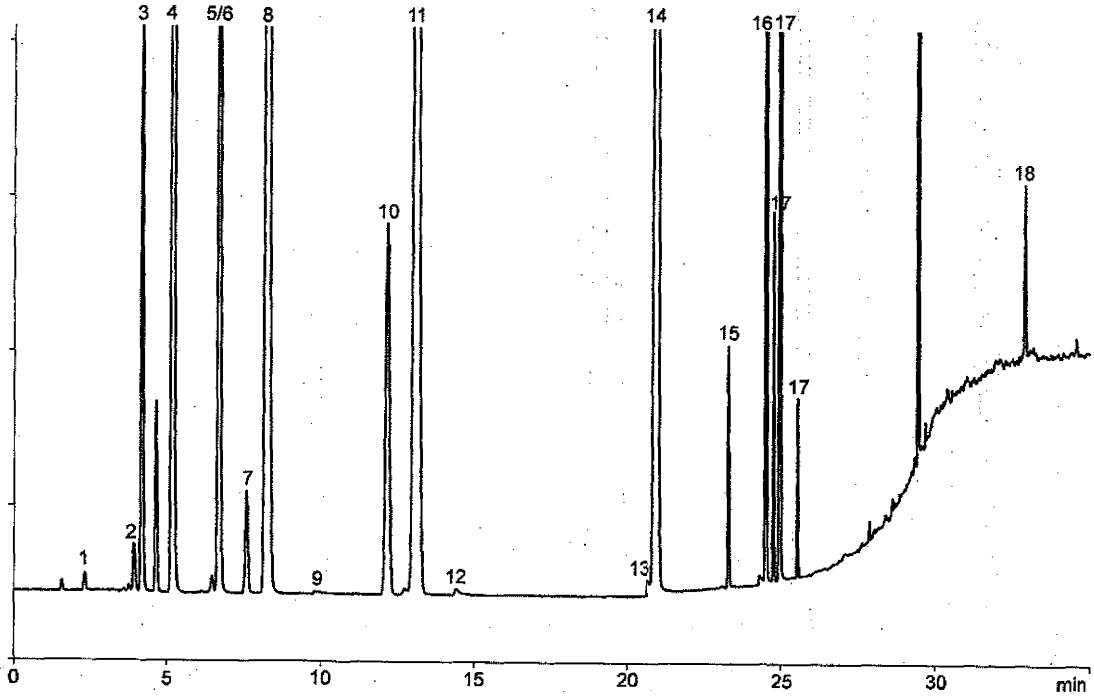
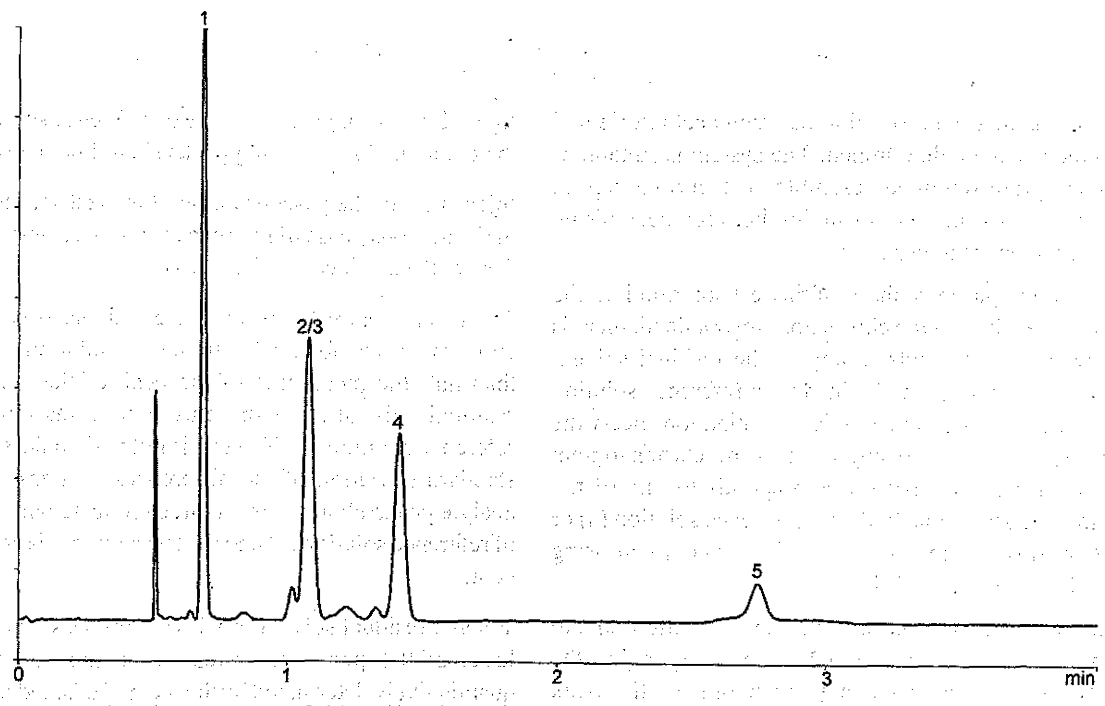


Fig.5.4-1



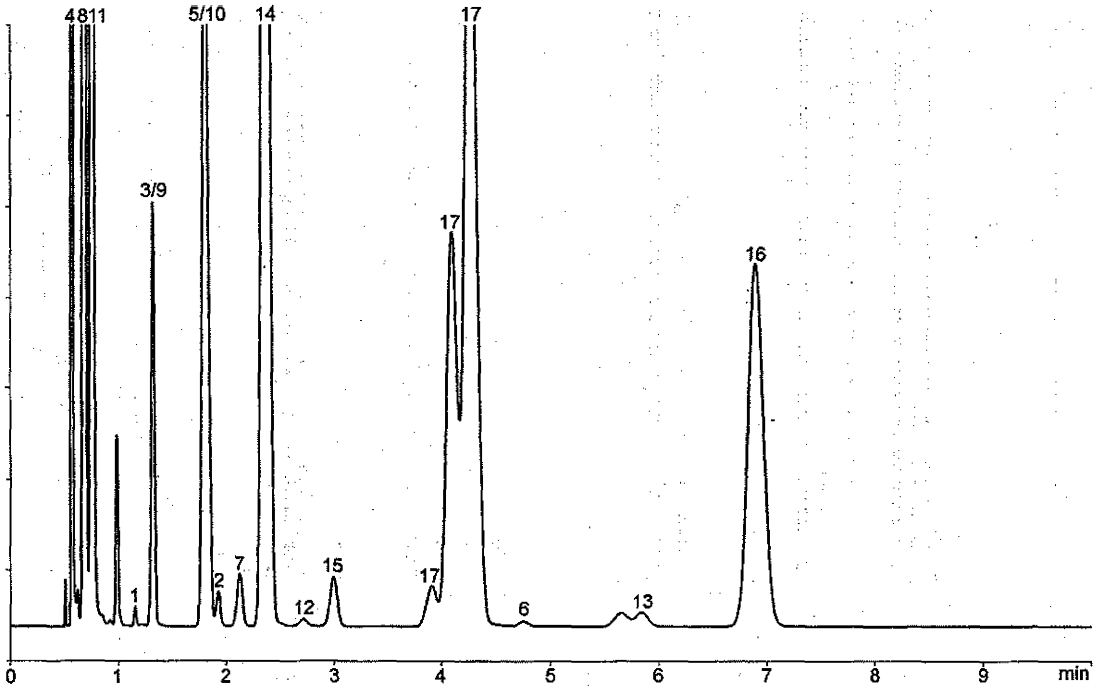
1. methanol
2. acetonitrile
3. dichloromethane
4. hexane
5. *cis*-1,2-dichloroethene
6. nitromethane
7. chloroform
8. cyclohexane
9. 1,2-dimethoxymethane
10. 1,1,2-trichloroethene
11. methylcyclohexane
12. 1,4-dioxan
13. pyridine
14. toluene
15. 2-hexanone
16. chlorobenzene
17. xylene *ortho, meta, para*
18. tetralin

Fig.5.4-2



1. 1,1-dichloroethene
2. 1,1,1-trichloroethane
3. carbon tetrachloride
4. benzene
5. 1,2-dichloroethane

Fig. 5.4-3



1. methanol
2. acetonitrile
3. dichloromethane
4. hexane
5. *cis*-1,2-dichloroethene
6. nitromethane
7. chloroform
8. cyclohexane
9. 1,2-dimethoxyethane
10. 1,1,2-trichloroethene
11. methylcyclohexane
12. 1,4-dioxan
13. pyridine
14. toluene
15. 2-hexanone
16. chlorobenzene
17. xylene *ortho, meta, para*
18. *tetralin* (t_R = 28 *min*)

Fig. 5.4-4

such conditions that the resolution between acetonitrile and trichloroethene can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Fig. 5.4-4 and the resolution between acetonitrile and trichloroethene is at least 1.0.

Inject 1 ml of the gaseous phase of the test solution into the column of system 2. If in the chromatogram obtained, there is no peak which corresponds to any of the residual solvent peaks in the chromatogram obtained with reference solution (a) or (b), then the substance under examination meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) and confirms the correspondence obtained when using system 1, then proceed as follows.

Inject 1 ml of the gaseous phase of reference solution (c) into the column of system 1 or system 2. If necessary, adjust the sensitivity of the system so that the height of the peak corresponding to the identified residual solvent(s) is at least 50 per cent of the full scale of the recorder.

Inject 1 ml of the gaseous phase of reference solution (d) into the column. No interfering peaks should be observed.

Inject 1 ml of the gaseous phase of the test solution and 1 ml of the gaseous phase of reference solution (c) into the column. Repeat these injections twice more.

The mean area of the peak of the residual solvent(s) in the chromatograms obtained with the test solution is not greater than half the mean area of the peak of the corresponding residual solvent(s) in the chromatograms obtained with reference solution (c). The test is not valid unless the relative standard deviation of the differences in areas between the analyte peaks obtained from three replicate paired injections of reference solution (c) and the test solution, is at most 15 per cent.

When a residual solvent of Class 2 or Class 3 is present at a level of 0.1 per cent or less, then the content may be quantitatively determined by the method of standard additions.

The entire procedure is shown in the Fig. 5.4-5 flow diagram.

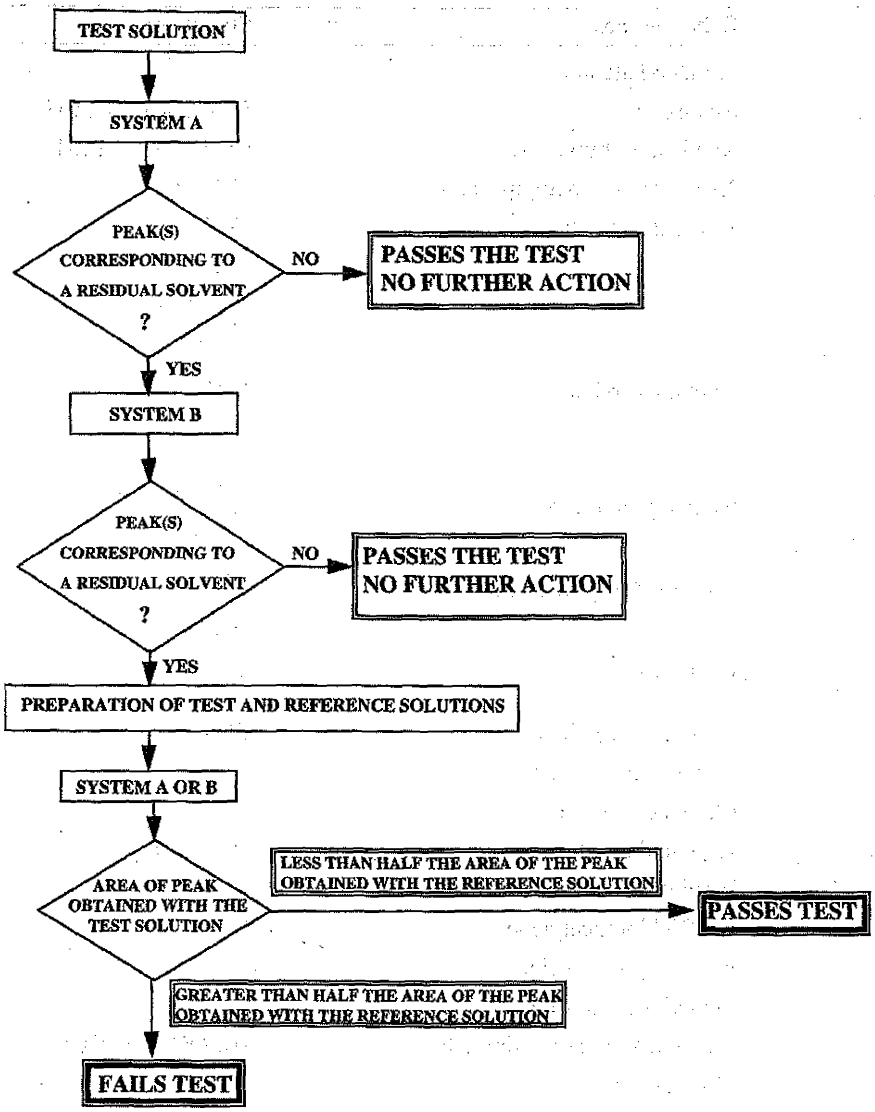
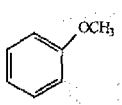
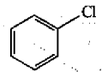
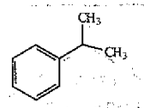
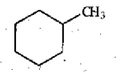
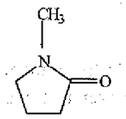

TEST SOLUTION
SYSTEM A
PEAK(S) CORRESPONDING TO A RESIDUAL SOLVENT ?
NO
PASSES THE TEST NO FURTHER ACTION
YES
SYSTEM B
PEAK(S) CORRESPONDING TO A RESIDUAL SOLVENT ?
NO
PASSES THE TEST NO FURTHER ACTION
YES
PREPARATION OF TEST AND REFERENCE SOLUTIONS
SYSTEM A OR B
AREA OF PEAK OBTAINED WITH THE TEST SOLUTION
LESS THAN HALF THE AREA OF THE PEAK OBTAINED WITH THE REFERENCE SOLUTION
PASSES TEST
GREATER THAN HALF THE AREA OF THE PEAK OBTAINED WITH THE REFERENCE SOLUTION
FAILS TEST

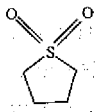
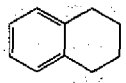
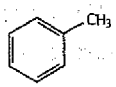
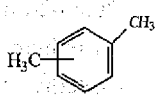
Fig. 5.4-5

Appendix 1. List of solvents included in the guideline

Solvent	Other Names	Structure	Class
Acetic acid	Ethanoic acid	CH_3COOH	Class 3
Acetone	2-Propanone	CH_3COCH_3	Class 3
Acetonitrile	Methyl cyanide	CH_3CN	Class 2
Anisole	Methoxybenzene		Class 3
Benzene	Benzol		Class 1
1-Butanol	*n*-Butyl alcohol Butan-1-ol	$CH_3[CH2]_3OH$	Class 3

Solvent	Other Names	Structure	Class
2-Butanol	*sec*-Butyl alcohol Butan-2-ol	$CH_3CH_2CH(OH)CH_3$	Class 3
Butyl acetate	Acetic acid butyl ester	$CH_3COO[CH_2]_3CH_3$	Class 3
Tert-Butylmethyl ether	2-Methoxy-2- methylpropane	$(CH_3)_3COCH_3$	Class 3
Carbon tetrachloride	Tetrachloromethane	CCl_4	Class 1
Chlorobenzene			Class 2
Chloroform	Trichloromethane	$CHCl_3$	Class 2
Cumene	Isopropylbenzene (1-Methylethyl)benzene		Class 2
Cyclohexane	Hexamethylene		Class 2
1,2-Dichloroethane	*sym*-Dichloroethane Ethylene dichloride Ethylene chloride	CH_2ClCH_2Cl	Class 1
1,1-dichlororoethene	1,1-Dichloroethylene Vinylidene chloride	$H_2C{=}CCl_2$	Class 1
1,2-Dichloroethene	1,2-Dichloroacetylene Acetylene dichloride	$ClHC{=}CHCl$	Class 2
Dichloromethane	Methylene chloride	CH_2Cl_2	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether Monoglyme Dimethyl cellosolve	$H_3COCH_2CH_2OCH_3$	Class 2
N,N-Dimethylacetamide	DMA	$CH_3CON(CH_3)_2$	Class 2
N,N-Dimethylformamide	DMF	$HCON(CH_3)_2$	Class 2
Dimethyl sulphoxide	Methylsulphinylmethane Methyl Sulphoxide DMSO	$(CH_3)_2SO$	Class 3
1,4-Dioxane	*p*-Dioxane [1,4]Dioxane		Class 2
Ethanol	Ethyl alcohol	CH_3CH_2OH	Class 3
2-Ethoxyethanol	Cellosolve	$CH_3CH_2OCH_2CH_2OH$	Class 2
Ethyl acetate	Acetic acid ethyl ester	$CH_3COOCH_2CH_3$	Class 3
Ethyleneglycol	1,2-Dihydroxyethane 1,2-Ethanediol	$HOCH_2CH_2OH$	Class 2

Solvent	Other Names	Structure	Class
Ethyl ether	Diethyl ether Ethoxyethane / 1,1'-Oxybisethane	$CH_3CH_2OCH_2CH_3$	Class 3
Ethyl formate	Formic acid ethyl ester	$HCOOCH_2CH_3$	Class 3
Formamide	Methanamide	$HCONH_2$	Class 2
Formic acid		$HCOOH$	Class 3
Heptane	*n*- Heptane	$CH_3[CH_2]_5CH_3$	Class 3
Hexane	*n*- Hexane	$CH_3[CH_2]_4CH_3$	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	$CH_3COOCH_2CH(CH_3)_2$	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	$CH_3COOCH(CH_3)_2$	Class 3
Methanol	Methyl alcohol	CH_3OH	Class 2
2-Methoxyethanol	Methyl cellosolve	$CH_3OCH_2CH_2OH$	Class 2
Methyl acetate	Acetic acid methyl ester	CH_3COOCH_3	Class 3
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	$(CH_3)_2CHCH_2CH_2OH$	Class 3
Methylbutylketone	2-Hexanone Hexane-2-one	$CH_3[CH_2]_3COCH_3$	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylethylketone	2-Butanone MEK Butan-2-one	$CH_3CH_2COCH_3$	Class 2
Methylisobutylketone	4-Methylpentane-2-one 4-Methyl-2-pentanone MIBK	$CH_3COCH_2CH(CH_3)_2$	Class 2
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	$(CH_3)_2CHCH_2OH$	Class 3
N-Methylpyrrolidone	1- Methylpyrolidine-2-one 1-Methyl-2-pyrolidinone		Class 2
Nitromethane		CH_3NO_2	Class 2
Pentane	*n*- Pentane	$CH_3[CH_2]_3CH_2$	Class 3
1- Pentanol	Amyl alcohol Pentan-1-ol Pentyl alcohol	$CH_3[CH_2]_3CH_2OH$	Class 3
1- Propanol	Propan-1-ol Propyl alcohol	$CH_3CH_2CH_2OH$	Class 3
2- Propanol	Propan-2-ol Isopropyl alcohol	$(CH_3)_2CHOH$	Class 3

Solvent	Other Names	Structure	Class
Propyl acetate	Acetic acid propyl ester	$CH_3COOCH_2CH_2CH_3$	Class 3
Pyridine	Aza benzene		Class 2
Sulfonane	Tetrahydrothiophene 1,1-dioxide		Class 2
Tetrahydrofuran	Tetramethylene oxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH_3CCl_3	Class 1
1,1,2-Trichloroethene	Trichloroethene	$HClC{=}CCl_2$	Class 2
Triethylamine	*N,N*-Diethylethanamine	$N(CH_2CH_3)_3$	Class 3
Xylene*	Dimethylbenzene Xylol		Class 2

* Usually 60 per cent m-xylene. 40 per cent p-xylene. 9 per cent of o- xylene with 17 per cent ethylbenzene.

5.5. Impurities

This chapter provides guidance on the control of impurities in drug substances and formulated preparations described in IP monographs. It applies mainly to totally synthetic organic medicinal substances and those substances obtained by synthetic modification of a naturally produced precursor. It is not necessarily relevant to other organic substances e.g., herbal products and crude products of plant or animal origin, biological and biotechnology products, oligonucleotides, radiopharmaceuticals, fermentation products and semi-synthetic products derived there from; inorganic substances and pharmaceutical excipients. Although the thresholds stated in this general chapter do not apply, the general concepts of reporting, identification (wherever possible) and qualification of impurities are equally valid for these classes. It provides an approach to the setting of limits for impurities in articles for which the individual monographs do not provide either a test or specific limits.

Impurities are critical quality attributes of drug substances and drug products because they have the potential to affect the safety and efficacy of the product. The monographs of the Pharmacopoeia have been designed to ensure the minimum acceptable quality of drug substances and drug products for users. Tests for related substances have also been provided in many monographs to limit impurities and degradation products. Although one of the primary objectives of the Pharmacopoeia is to guarantee the identity, strength, purity and quality of official articles, it is not possible to include in each monograph a test for every impurity or contaminant or even an adulterant that might be present. The exclusion of a limit for impurities in a monograph does not absolve the manufacturer of providing assurance to the user on the safety

of a drug. It is incumbent on the manufacturer to follow Good Manufacturing Practices (GMP) and to ensure the limitation of impurities based on knowledge of the properties of the chemical entity and the likelihood of related substances being associated with the end product during production and subsequent storage.

Impurity measurements for drug products using chromatographic methods could present a challenge to the Pharmacopoeial standards setting due to low concentrations of the impurity and complexity of matrix. As a consequence, some of the monographs for Pharmacopoeial preparations rely on chromatographic assays. Where more significant impurities are known, some monographs set forth specific tests for these impurities.

In general, the tests in a monograph are tests for purity that provide information on the extent of known potential or actual impurities rather than for guaranteeing freedom from all possible impurities. The tests are not necessarily designed to detect any foreign contaminants or adulteration. Material found to contain an impurity not detectable by the prescribed tests of a monograph may be deemed to be not of Pharmacopoeial quality particularly if the nature of the impurity (ies) found is not compatible with GMP.

Chemical tests that limiting the levels of particular impurities or classes of impurities are often augmented by physical tests such as specific optical rotation, absorbance, melting point, clarity and colour of solution and for liquids, refractive index, boiling point range and weight per ml etc. Besides, non-specific tests such as sulphated ash, loss on drying etc. contribute to the assurance of the general quality of the article and of the use of GMP in its production, the avoidance of contamination especially by inorganic substances and the removal of volatile solvents.

Notwithstanding this situation, there is a need to limit impurities that may arise from various sources in the course of manufacture.

Classification of Impurities

Impurities can be classified into the following categories:

— Organic impurities
— Inorganic impurities
— Residual solvents

Organic impurities. Organic impurities may be drug-related or process-related and consist of identified, specified impurities, unidentified, specified impurities or total unknown impurities and are subject to control for all drug substances and drug products. In designing the specifications for any drug substance, a manufacturer should determine the actual and potential impurities most likely to arise during the synthesis, purification and storage, on the basis of scientific knowledge of the chemical reactions involved in the synthesis. Organic impurities can arise during the manufacturing process and or storage of the drug substance include starting materials, intermediates, by-products, degradation products, reagents and ligands.

Inorganic impurities. Inorganic impurities usually result from the manufacturing processes and include catalysts, reagents, elemental impurities and other residual metals, inorganic salts and other materials e.g., filter aids, charcoal. Impurities associated with input raw materials and storage conditions can also contribute to the impurity profile of the drug substance. The detection and quantification of such impurities by classical physicochemical methods should not present any problems. Elemental impurities can include catalysts and environmental contaminants that may be present in drug substances. These impurities may occur naturally, be added intentionally, or be introduced inadvertently (e.g., by interactions with processing equipment and the container–closure system). When heavy metals or elemental impurities are known to be present, have been added, or have the potential for, can be controlled with appropriate limits as specified in chapter 2.3.13. Heavy Metals or 5.10. Elemental Impurities respectively.

Residual solvents. Residual solvents are inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions during the synthesis of a drug substance. Since these are generally of known toxicity, they can be controlled with appropriate limits as listed in chapter 5.4. Residual Solvents. In addition to a general limit on solvents remaining behind in the final drug substances, some drugs need specific limits for specific solvents, where variation in levels of known solvents requires control e.g. methanol in Gentamicin Sulphate.

Irrespective of the nature of these impurities or degradation products, limits and acceptance criteria in drug substances are based on chemistry and safety concerns and have to be worked out on the basis of factors such as toxicity, process capability, manufacturing practices and so on. Elemental impurity limits for a drug substance may be required depending on the outcome of the risk assessment for the finish product. The basic principle for setting limits is that levels of impurities in a drug substance must be controlled throughout its development to ensure its safety and quality for use in a drug product. Documented evidence that the analytical procedure used to evaluate impurities is validated and suitability for the detection and quantitation of impurities should be established.

Organic Impurities

Test methods

It is usual to include a test for related substances in a monograph for a medicinal substance or product. These may be manufacturing impurities intermediates or by-products) or

degradation products or both. When preparation of a monograph is initiated, the manufacturer is asked to provide information concerning the nature of such impurities, the reason for their presence, the amounts that may be encountered in material prepared under conditions of Good Manufacturing Practice (GMP) and the manner in which proportions may vary on storage, together with an indication of the toxicity of any impurities in relation to that of the substance itself. Where there is only one manufacturer of a substance, Pharmacopoeial limits are set in the knowledge that the level of impurities in production batches of the substance will have been accepted by the registration authority after a full consideration of the toxicity studies and clinical trials carried out before the granting of a license. Such studies and trials will have been carried out on material with an impurity profile that is qualitatively and quantitatively similar to that of subsequent production batches. Any subsequent changes to the manufacturing process by the original manufacturer or the introduction of material from another manufacturer utilizing a different route of synthesis will be subject to the need to demonstrate essential similarity or to provide equivalent data to the regulatory authority. In some cases a change in production or source may give rise to impurities that are not adequately controlled by the Pharmacopoeial monograph. Appropriate revisions of the monograph will be carried out provided that the Pharmacopoeial authority is notified of the need and that it is supplied with the relevant information

The most widely used methodology is chromatography which is the basis of the test for related substances. The test may be specific or general. Specific tests may be supplemented by a more general test controlling other impurities. A specific test is one where a particular impurity arising from the manufacturing process or from degradation needs to be limited on grounds of toxicity or any other special reason. Where the impurity is known to be particularly toxic, this should be taken into account. Such specific tests include a chromatographic or colorimetric comparison with a sample of the named substance e.g. salicylic acid in aspirin. Both types of tests require the use of Reference substances. In chromatographic determinations, in the absence of a reference substance it is usual practice to limit the level of impurities by the simple test of comparison of the unknown spot or peak with a spot or peak obtained with a dilute solution of the substance under examination.

Meaningful limitation of impurities is possible only with validated analytical methods that can help in determining the limits of detection and quantitation. With drug products the methods should be validated to demonstrate that the drug product components and impurities unique to the drug substance and excipients do not interfere with or are separated from specified and unspecified degradation products in the final product.

Thin-layer chromatography (TLC) is quick and is particularly useful in process monitoring and in detecting impurities during the course of manufacture. However, it has its limitations in fixing limits for specific impurities in the final product although for long it was the most widely used for this purpose.

Total impurities can be determined by gas chromatographic and liquid chromatographic tests, where the total impurity levels can be obtained by summation of the peak areas due to related substances (usually in the range 1 to 2 per cent) are applicable till the end of the shelf-life. Here again, this procedure is rarely adopted in TLC tests because of the semi-quantitative nature of estimating individual spots and the imprecise nature of expressing results for the total impurities. This drawback can be overcome to an extent by the use of two- and three- level tests. In the former, in addition to a nominal concentration of the reference solution, another at a lower concentration is used for spotting the plate; in the latter, two more solutions at different lower concentrations are used.

In liquid chromatographic tests the relative detector correction factor that expresses the sensitivity of a detector relative to a standard substance is an important factor to be considered. As a general thumb rule, if the correction factor of an impurity is between 0.8 and 1.2, it may be considered the impurity has a similar response to that of the drug substance. Also, correction factors less than 0.2 or more than 5 are not recommended. In such cases, the method needs to be amended to bring the correction factor within the acceptable range by either choosing a different wavelength of measurement or a different method of visualisation.

Unknown impurities may be limited by reference to a dilution of the substance under examination used as a reference solution.

Manufacturers shall develop acceptance criteria for impurities that are justified by appropriate safety considerations and consistent with current applicable regulatory guidances. Impurities known or suspected to be unusually toxic (e.g., mutagenic impurities) or to produce undesired pharmacological effects in drug substances and drug products, require more stringent control compared to non-mutagenic impurities and therefore, in such cases, the limit of detection and limit of quantitation of the analytical procedures shall be commensurate with the current applicable regulatory guidances and the acceptance criteria to ensure patient safety.

Identification of peaks is generally not based on absolute retention times since these may be too 'system dependent'; however, advice such as 'the principal peak has a retention time of about 'x' minutes may be given. Although in some cases, where a simple chromatogram is expected to show a limited number of impurities, an expected relative retention time may be given to designate impurities.

Reporting of Impurities

The reporting threshold can be established using current applicable regulatory guidance's or other acceptable scientific means. Impurities presenting above the reporting threshold shall be reported according to the relevant analytical procedure. Impurity results shall be reported as numerical values and rounded according to conventional rules (see General Notices, Rounding Rules for Calculation of Results). All impurities at a level more than (>) the reporting threshold shall be summed and reported as a value for total impurities, unless otherwise indicated in the monograph.

Identification of Impurities

Impurities present above the identification threshold for drug substances and drug products at release and on storage shall be investigated and all reasonable attempts shall be made to identify these impurities. The identification threshold can be established using Table 1 and Table 2. Lower thresholds may be required for impurities known or suspected to be unusually toxic (e.g., mutagenic impurities) or to produce undesired pharmacological effects. Higher thresholds may be applied if scientifically justified according to ICH Q3A and ICH Q3B.

Qualification of Impurities

Qualification is the process of establishing the biological safety of impurities at the specified level(s). Qualification of impurities shall be based on a combination of factors including safety, intended use, applicable guidances, and scientific rationale.

Acceptance criteria for Impurities

Acceptance criteria shall be set for all impurities present above the reporting thresholds for drug substances and drug products and taking into account the qualification (the acquisition and evaluation of data establishing the safety of an impurity) of the degradation products, accelerated and long-term stability data, the expected expiry period and the recommended storage conditions for the drug product. Allowance should be made for the normal variations in manufacturing, analysis and the stability profile.

Specified impurities/degradation products can be either identified or unidentified. Specified identified impurities/ degradation products should be included along with specified unidentified /degradation products estimated to be present at a level more than (>) the identification threshold. Unspecified impurities/degradation products are limited by a general acceptance criterion, which is Not More Than (≤) the identification threshold and Not Less Than (>) the reporting threshold. A rationale for the inclusion or exclusion of impurities in the specification should be documented.

The acceptance criteria shall be based on applicable guidances or other acceptable scientific means, with safety as the primary consideration and not solely based on process capability. If the individual monograph does not include a procedure for quantifying an impurity or acceptance criterion for an observed impurity, the manufacturer is responsible for developing and validating analytical procedures and then establishing appropriate acceptance criterion. In the case of a complex impurity profile, it may not be feasible to resolve each of the impurities individually or to detect them and quantify them using a single analytical procedure. In such cases manufacturers should consider alternate approaches such as the use of multiple analytical procedures to test for impurities or acceptance limits may be established based on grouping of impurities, as appropriate. Similar principles may be applied to set thresholds and acceptance criteria for degradation products in over-the-counter (OTC) monograph drug products and should be reported, identified and/or qualified. Measurement of degradation products can be challenging for products containing multiple drug substances and complex formulations. The use of placebo as controls may aid in the deconvolution of chemical changes in stability studies that could be related to excipients rather than the drug substance.

It is recommended that the specifications for a drug substance should include the acceptance criteria for the following, where applicable,

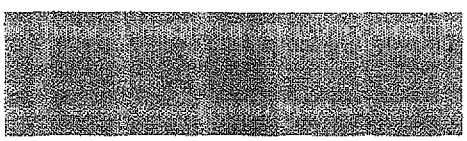
- each specified identified impurity,
- each specified unidentified impurity,
- any unspecified impurity with acceptance criteria of not more than the identification threshold,
- total impurities
- inorganic impurities (Controlled as per 2.3.13. Heavy metals or 5.10 Elemental impurities)
- residual solvents (Controlled as per 5.4. Residual solvents)

Unless otherwise indicated, total impurities in the drug substance monographs are the sum of all specified (identified and unidentified) and unspecified impurities above the reporting threshold.

The specifications for a drug product should include, acceptance criteria for the following, where applicable,

- each specified identified degradation product
- each specified unidentified degradation product
- any unspecified degradation product with an acceptance criterion of Not More Than the identification threshold
- total degradation products

Unless otherwise indicated, total degradation products in the drug product monographs are the sum of all specified and unspecified degradation products above the reporting threshold.



In Indian Pharmacopoeia, the preferred terminology applicable for organic impurities (specified and unspecified impurities/ Degradation Products) is "any secondary peak", any other secondary peak and "sum of all the secondary peaks" in the drug substance and drug product monographs,. There are several scenarios for citing specified organic impurities in an IP monograph. Specified impurities/ degradation products can be either identified or unidentified. They are listed and limited with a specific acceptance criterion in a monograph. For identified impurities/degradation products, the chemical name is provided. Unidentified impurities /degradation products are named with a general designation along with the product name e.g., Impurity A, Impurity B. In all the cases "Any secondary peak" and "Any other secondary peak" correspond to unspecified impurities. Total impurities in the drug substance/ drug product monograph are the sum of all specified and any other secondary peak above the reporting threshold i.e., ignore any peak less than certain value or disregard limit. Typically, disregard limit covered by a monograph is set in accordance with the reporting threshold as mentioned above under Organic impurities.

In some cases, drug substance process-related impurities are also detected in the drug product, if appropriate, and limited by appropriate acceptance criteria. Drug substance process-related impurities detected in the drug product and included in its specification may include a note that certain drug substance process-related impurities are listed only for information and should not be included in the total degradation products. When this note is included, the total impurities/ degradation products should only include all specified and unspecified impurities/degradation products above the reporting threshold, with the exception of these designated process- related impurities.

In chromatographic tests, during quantitation, peaks caused by solvents and reagents or arising from the mobile phase or sample matrix shall be disregarded, as well as other peaks that the monograph explicitly states are to be disregarded. During quantitative determination of an impurity in liquid and gas chromatographic tests, choice of an appropriate threshold setting and appropriate conditions for the integration of the peak areas is important. Principles from 2.4.14, 2.4.13 and 2.5.10 such as signal-to-noise ratio, quantification limits, and quantitation methods, peak separation between impurities and the substances under examination should be observed to confirm that performance of the system is satisfactory. The quantitation limit for the analytical procedure should not be more than the reporting threshold.

Acceptance criteria for impurities (including unusually toxic, for example, mutagenic impurities) should be supported by appropriate toxicological evaluation. Lower thresholds may be required for impurities known or suspected to be unusually toxic.

The entire procedure for decision on Organic Impurities is shown in Fig. 5.5-1

Organic Impurities in Drug Substances

Organic impurities in drug substances arising from the manufacturing process and/or storage should be controlled. A rationale for the inclusion or exclusion of impurities in the specification should be documented. The organic impurities to be controlled in the drug substance are the process-related impurities and degradation products. They can be identified or unidentified. Process-related impurities are generated during drug substance manufacturing, such as by-products, residual starting materials, intermediates, reagents, and ligands. Impurities that increase over time on storage are considered degradation products.

When there are changes to the chemistry, manufacturing, and/ or controls of the drug substance (e.g., a different manufacturer or manufacturing site, scale/equipment, starting materials, synthetic pathways, and/or purification steps) described in a monograph, they should be evaluated to determine if the differences affect the impurity profile listed in the existing monograph.

Unless otherwise prescribed or justified and authorised organic impurities in a drug substance intended for human and veterinary use, are to be reported, identified where possible, and qualified as indicated in Table 1. Because toxicity is a dose-related phenomenon, the thresholds are set based on the amount of drug substance administered per day. Lower thresholds can be appropriate if the impurity is unusually toxic or produces undesirable pharmacological effects.

Organic Impurities in Drug Products

Usually, the organic impurities to be controlled in the drug product are only the degradation products resulting from the degradation of the drug substance or the interaction of the drug substance with excipients and/or the primary packaging configuration. They can be identified or unidentified. A rationale for the inclusion or exclusion of impurities in the specification should be documented. Drug substance process-related impurities need not be monitored or specified in drug products unless they are also degradation products.

Unless otherwise prescribed or justified and authorised organic impurities in a drug products intended for human and veterinary use, are to be reported, identified where possible, and qualified as indicated in Table 2. Because the toxicity is dose-related, the thresholds are based on the amount of drug substance administered per day. The amount of drug substance administered per day is based upon the manufacturer's maximum recommended labelled dosage per day. Lower thresholds can be appropriate if the degradation

product is unusually toxic or produces undesirable pharmacological effects.

In cases of complex impurity profiles, limits may be established based on the grouping of impurities, if appropriate and scientifically justified. For drug products that contain multiple drug substances, degradation products from each active ingredient should be controlled. Manufacturers should provide rationale and supporting data to justify the acceptance criteria for impurities associated with each drug substance, as applicable.

For DNA reactive impurities, the requirements of ICH guideline M7 Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk must be complied with for active substances to be used in medicinal products for human use, in cases defined in the scope of the guidelines.

The entire procedure for decision on Identification and Qualification of an Impurity in drug substance or a Degradation Product in drug product is shown in Fig. 5.5-2.

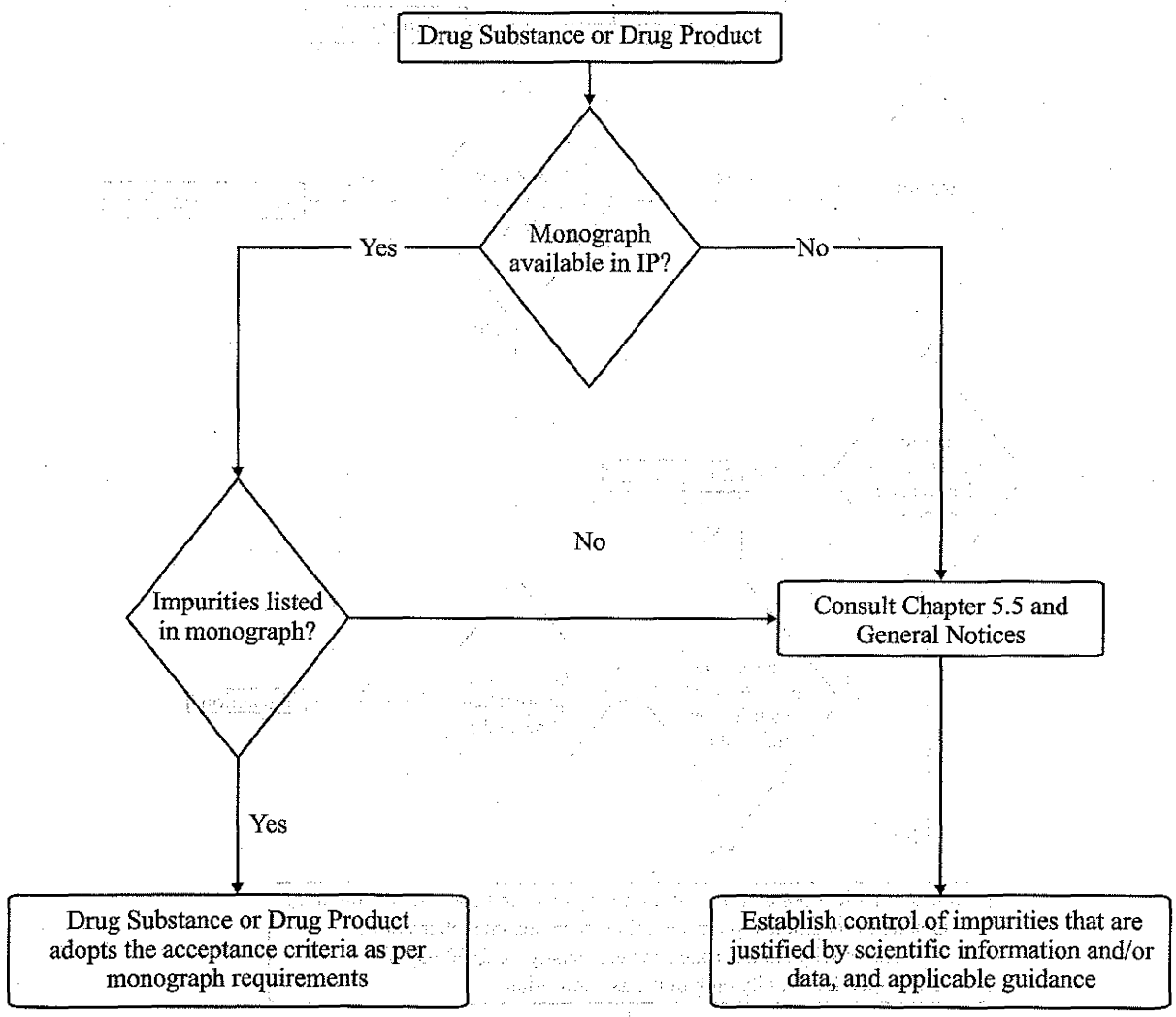

Drug Substance or Drug Product
Monograph available in IP?
Yes
No
Impurities listed in monograph?
No
Consult Chapter 5.5 and General Notices
Yes
Drug Substance or Drug Product adopts the acceptance criteria as per monograph requirements
Establish control of impurities that are justified by scientific information and/or data, and applicable guidance

Fig. 5.5-1: Procedure for decision on organic impurities

NOTE —All impurities specific to a given drug product formulation may or may not be included in the IP. If the impurity is listed in the monograph, follow monograph limits. If the impurity is not listed, consult General chapters 5.5 and General Notices for guidance. Non-monograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

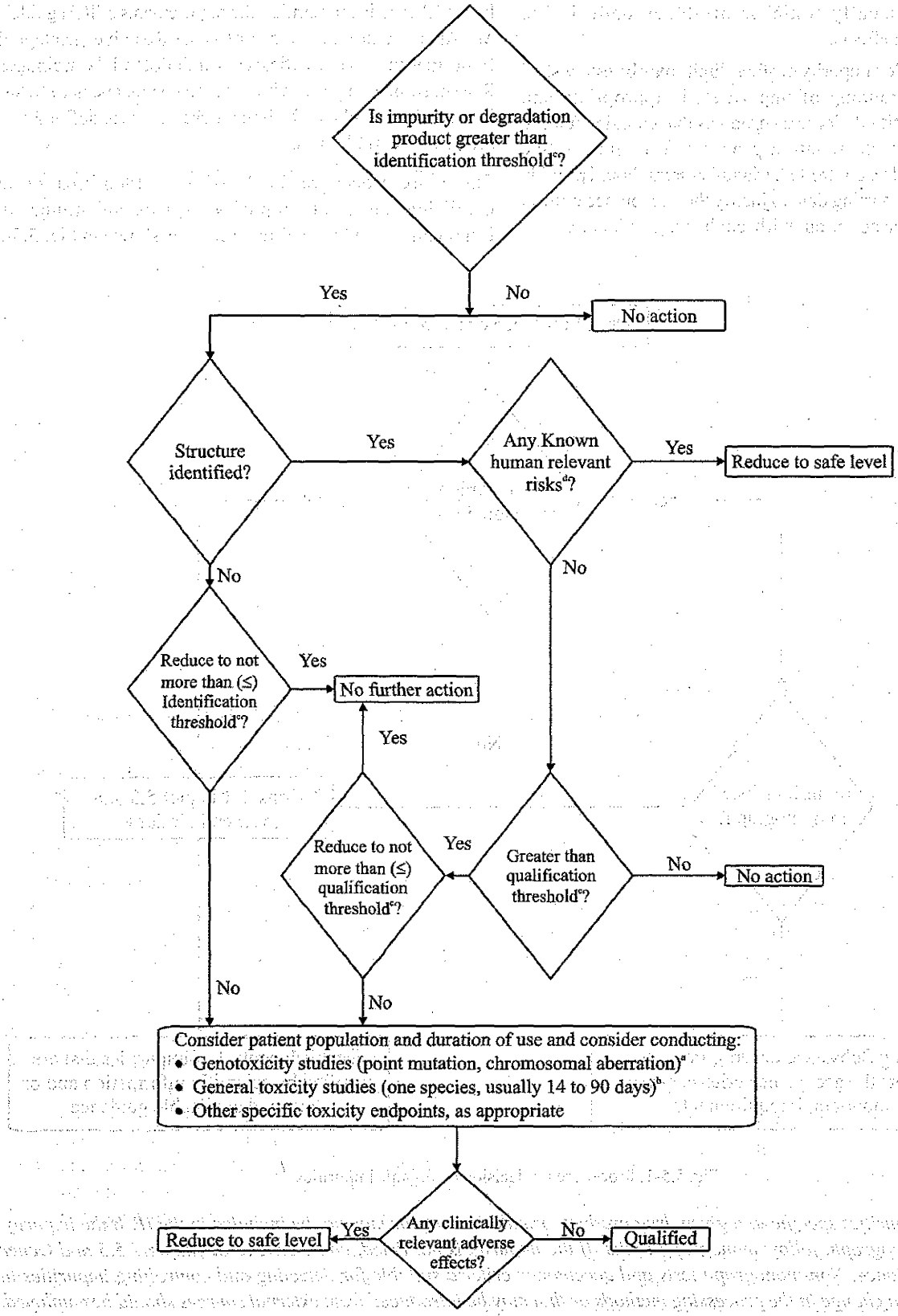


Fig. 5.5- 2: Procedure for decision on Identification and Qualification of an Impurity in drug substance or a Degradation Product in drug product

NOTE —

a) *If considered desirable, a minimum screen (e.g., genotoxic potential), should be conducted. A study to detect point mutations and one to detect chromosomal aberrations, both in vitro, are considered an appropriate minimum screen.*

b) *If general toxicity studies are desirable, one or more studies should be designed to allow comparison of unqualified to qualified material. The study duration should be based on available relevant information and performed in the species most likely to maximise the potential to detect the toxicity of an impurity or degradation product. On a case-by-case basis, single-dose studies can be appropriate, especially for single-dose drugs. In general, a minimum duration of 14 days and a maximum duration of 90 days would be considered appropriate.*

c) *Lower thresholds can be appropriate if the impurity or degradation product is unusually toxic.*

d) *For example, do known safety data for this impurity/degradation product or its structural class preclude human exposure at the concentration present?*

Table 1 – Reporting, identification and qualification of organic impurities in Drug substances

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Human or human and veterinary use	≤ 2 g	0.05 per cent	0.10 per cent (1.0 mg)[a]	0.15 per cent (1.0 mg)[a]
Human or human and veterinary use	>2 g	0.03 per cent	0.05 per cent	0.05 per cent
veterinary use only	Not Applicable	0.1 per cent[1,2]	0.2 per cent[2]	0.5 per cent[2]

[a]Total daily intake (TDI; in parentheses) applies if it is lower than the calculated value.

[1]Higher reporting thresholds should be scientifically justified.

[2]Lower thresholds can be appropriate if the impurity is unusually toxic.

Table 2 – Reporting, identification and qualification of organic impurities or degradation products in Drug products

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Human use	< 1 mg	0.1 per cent	1.0 per cent (5 µg)[a]	1.0 per cent (50 µg)[a]
	≥ 1 to < 10 mg	0.1 per cent	0.5 per cent (20 µg)[a]	1.0 per cent (50 µg)[a]
	10 mg	0.1 per cent	0.5 per cent (20 µg)[a]	0.5 per cent (200 µg)[a]
	> 10 – 100 mg	0.1 per cent	0.2 per cent (2 mg)[a]	0.5 per cent (200 µg)[a]
	> 100 mg – 1 g	0.1 per cent	0.2 per cent (2 mg)[a]	0.2 per cent (3 mg)[a]
	> 1 – 2 g	0.05 per cent	0.2 per cent (2 mg)[a]	0.2 per cent (3 mg)[a]
	> 2 g	0.05 per cent	0.10 per cent	0.15 per cent
Veterinary use	Not Applicable	0.3 per cent[1]	1.0 per cent[1]	1.0 per cent[1]

[a]Whichever is lower, calculated value or TDI (in parentheses).

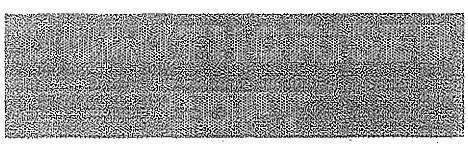
[1]Higher threshold should be scientifically justified.

Definition of Terms

Any secondary peak The terminology used in IP monographs for organic impurities i.e., specified (identified or unidentified) and unspecified impurities/ degradation Products above the reporting threshold.

Degradation Product An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the new drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and or the immediate container closure system.

Degradation Profile	A description of the degradation products observed in the drug substance or drug product.
Drug substance process-related impurity	An impurity generated during drug substance manufacturing. Process-related impurities may include starting materials, by-products, intermediates, reagents, ligands, and catalysts. In this chapter, it is also called "process impurity".
Enantiomeric Impurity	A compound with the same molecular formula as the drug substance that differs in the spatial arrangement of atoms within the molecule and is a non-super imposable mirror image.
Identification threshold	A limit above which an impurity or degradation product should be identified.
Identified impurity/ Degradation Product	An impurity or degradation product for which a structural characterisation has been achieved.
Impurity profile	A description of the identified and unidentified impurities present in a drug substance or drug product.
Impurity	An impurity is any component of a drug substance that is not the chemical entity defined as the drug substance, and for a drug product, any component that is not a formulation ingredient.
Nominal Concentration	Concentration calculated on the basis of the concentration of the prescribed reference and taking account of the prescribed correction factor.
Other detectable impurities	Potential impurities with a defined structure that are known to be detected by the tests in a monograph but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities. They are unspecified impurities and are thus limited by a general acceptance criterion.
Potential impurity	An impurity that theoretically can arise during manufacture or storage. It may or may not actually appear in the drug substance.
Qualification	The process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified.
Qualification threshold	A limit above which an impurity or degradation product should be qualified.
Related substances	Title used in monographs for general test for organic impurities.
Reporting threshold or disregard limit	A limit above which an impurity or degradation product should be reported. Reporting threshold is the same as reporting level in ICH Q2B. The existing related term in IP monographs appears as "ignore any peak". Peak responses must be corrected by multiplying the correction factor when the information is provided in the individual monograph. In chromatographic tests, the nominal content at or below which peaks/ signals are not taken into account for calculating a sum of impurities. The numerical values for the disregard limit and the reporting threshold are usually the same.
Specified impurity/ Degradation Product	An impurity or degradation product that is individually listed and limited with a specific acceptance criterion in a monograph. A specified impurity can be either identified or unidentified.
Total impurities/ total degradation product	In a drug substance/ drug product monograph, "total impurities/ degradation products" are the sum of all specified and unspecified impurities/ degradation products above the reporting threshold.
Unidentified impurity/ unidentified degradation product	An impurity for which a structural characterisation has not been achieved and that is defined solely by qualitative analytical properties (for example, chromatographic retention or relative retention times).
Unspecified impurity/ Degradation Product	Any impurity/degradation product that is not individually listed with its own specific acceptance criterion is considered "unspecified" and is limited by a general acceptance criterion.



Synonyms for "unspecified impurity/ degradation product" include "other unspecified impurity/degradation product/other secondary peak" or "other individual unspecified impurity/ degradation product/any other secondary peak".

5.6. Water for Pharmaceutical Use

Water is one of most widely and abundantly used substances in pharmaceutical manufacturing. It is required for a variety of purposes ranging from manufacturing processes to the preparation of the final dosage forms. The quality of water therefore assumes considerable importance.

This chapter is not exhaustive in scope; it contains points that are basic information to be considered, when appropriate, for the processing, holding and use of water.

The control of the chemical and microbiological quality of water for pharmaceutical use is governed by many factors of which the most important is the variability of the basis source viz. municipal water or any other water. The starting material for most forms of water is drinking water which should normally be subject to municipal or any other local regulations or is drawn from a private well or reservoir. Water prepared from other starting material may have to be processed to meet drinking water standards. Drinking water itself may be used in the manufacture of drug substances but not in the preparation of dosage forms, or in the preparation of reagents and test solutions.

There are many different grades of water for pharmaceutical purposes. Broadly, there are two types of water: bulk waters, typically produced on site and packaged waters which are produced, packaged, and sterilised to preserve microbial quality throughout their shelf life.

Given below is a brief description of the various types of pharmaceutical waters and their significant uses or attributes.

Drinking Water. Drinking water can be referred to as Potable water. Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the allowed contaminant levels in Drinking Water are generally considered safe for use for official substances and other drug substances. Where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing process, perhaps even as pure as Water for Injection or Purified Water. Such higher purity waters, however, might require only selected attributes to be of higher purity than Drinking Water.

Drinking water is the prescribed source or feed water for the production of pharmaceutical grade waters. It may be derived from a variety of raw water sources including a public water utility, private water supply or a combination of these sources. It must comply with the chemical and microbiological quality attributes of either BIS drinking water specification or WHO drinking-water guidelines or Schedule-M of the Drug & Cosmetics Rules 1945 or U.S. EPA National Primary Drinking Water Regulations.

There shall be validated system for treatment of water drawn from own or any other source to render it potable in accordance with standards specified by the BIS or other Drinking water regulations, as the case may be, So as to produce Purified water confirming to Pharmacopoeial specification. The potentially objectionable chemical species of certain organic and inorganic contamination should not be present more than standard limits. Typical processes employed for the production of Drinking water from raw water includes filtration, softening, disinfection, iron removal, precipitation and reduction of specific inorganic/organic materials. Drinking water shall be stored in tanks, which do not adversely affect quality of water and ensure freedom from microbiological growth. The tank shall be cleaned periodically and records maintained by the licensee in this behalf.

Drinking water shall comply with the BIS physicochemical test specifications such as pH, chlorides, total hardness, toxic substances, radioactive substances, pesticide residues etc. The Drinking water shall be free from pathogenic microscopic organisms. Microbiological requirements of Drinking water ensure the absence of coliforms, which, if determined to of fecal origin, may indicate the potential presence of other potentially pathogenic microorganisms and viruses of fecal origin. The Drinking water must not be detect any *Escherichia coli* or Total Coliform Bacteria in 100 ml and Total aerobic microbial count should not be more than 500 colony-forming units (CFU) per ml. Drinking water free from individual pathogenic microorganisms such as *Escherichia coli, Salmonella, Staphylococcus aureus and Pseudomonas aeruginosa* per 100 ml sample shall be used for the preparation of Purified water.

Purified Water. This article is used as an excipient in the production of nonparenteral preparations and in other applications such as cleaning of equipment and non-parenteral product-contact components. Unless otherwise specified, it is also to be used for all tests and assays of the Pharmacopoeia.

in a particular laboratory, unless the sources of variation are the same as occurring in the given estimates.

Potency estimates are generally made on the basis of the logarithms of the doses (see Section 5.7.5)

When the terms 'stated' potency and 'estimated' potency are used in this section and elsewhere, the following definitions are intended.

Stated potency —This is a nominal value assigned to a formulation or preparation from knowledge of the potency of the bulk material or, in the case of bulk drugs, it may be a calculated potency.

Estimated potency — This is the potency calculated from the data obtained from assays.

5.7.3. Glossary of Symbols

Symbol	*Definitions*
b	estimate of slope of regression line of response on log dose based on all preparations in the assay.
c′	constant used in evaluation of *fiducial* limits (Table 7)
d	number of dose levels for each preparation in a balanced assay
f	degrees of freedom which is the number of independent observations in a set of data.
h	number of preparation in an assay, including the standard preparation.
k	number of different treatments within an assay (k = dh)
n	number of replicates in each treatment.
n′	number of individual potency estimates
s^2	estimate of variance given by error mean square in analysis of variance — also used with a letter subscript, e.g. S_M^2 is variance of log potency M
s	estimate of standard deviation = square root of s^2
s_1, s_2	low and high doses of standard preparation S measured in units of activity or weight.
t	student's statistic (Table 1)
$u_1,...,z_3$	doses of test preparation U .....Z measured as specified in the monographs
w	weighing co-efficient in probit analysis (Table 16)
y	individual response or transformed response.
y′	calculated response to replace as missing value.
$\bar{y}_s,...,\bar{y}_Z$	mean responses for standard and unknown preparations
A_U	potency assumed for test preparation U when making up doses.
$B_1....B_2n$	total response for each subject (1 to 2 n) in twin cross-over assay.
B′	incomplete total response in a block or row containing one missing value.
C	statistic used in calculation of fiducial limits (Equation 17)
D_I, D_{II}	total response on day I or day II in a cross –over assay
E	regression sum of squares (Table 4)
F	ratio of two independent estimates of variance (Table 6)
G′	incomplete total response in an assay , excluding a missing value.
I	interval between adjacent log doses
K	correction term used in analysis of variance $\left[K = \frac{(\Sigma y)^2}{N}\right]$
L	fiducial interval in logarithms
Ls,...,Lz	linear contrasts for standard and unknown preparations (Tables 3 and 4)
M	estimate of log potency — used with a letter subscript in a multiple assay to denote a particular preparation (M = log R)
$\overline{M}$	mean of several independent estimates of M
M′	log potency ratio or estimate of log potency before correction by assumed potency (M′ = log R′)
N	total numbers of responses in an assay
Ns, Nu	total numbers of responses for preparation S and U
P	probability
R	potency estimate — used with a letter subscript to denote a particular test preparation (R = antilog M)
R′	estimate of potency or potency ratio before correction by assumed potency (R′ = antilog M′)
$R_1,...,Rn$	total response in each block of a randomized block design
S	standard preparation
S	total response to a standard preparation
S1, S2	total responses to low and high doses of standard preparation S

T′	incomplete total response to treatment, excluding a missing value
U,...,Z	unknown preparations under test
U,...,Z	total responses to test preparation U,...,Z
U_1, U_2	total responses to low and high doses of unknown preparation U
W	statistical weight used in combination of several independent estimates of log potency.
X	log dose – also used with subscript to indicate a particular preparation, e.g. X_S
$\overline{X}$	mean log dose
X^2	chi-square statistic

NOTE — All logarithms throughout this section are to base 10.

5.7.4. Randomisation

The allocation of individual units (animals, tubes, etc.) to different experimental groups should be made by some strictly random process. Any other choice of experimental conditions, which is not deliberately allowed for in the experimental design, should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered. In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be made from computer generated design or from standard tables of random sampling numbers which are usually accompanied by instructions for use.

5.7.5. Estimates of Error from Repeated Assays along with Illustrative Examples

When the same preparation has been assayed a number of times by exactly the same method and with the same number of observations in each assay, or when a number of preparations have been assayed more than once by the same method, the error of a single estimate can be calculated from the differences occurring among the potency estimates. The resulting error is, of course, applicable only to assays of the same type and size as those used in the calculations. This method of calculation has the advantage that it gives a direct estimate which takes all sources of random error into account. It has the disadvantages that it does not give a reliable value for the error unless a large number of results are available, and that its validity depends on the assumptions that the error is normally distributed and remains constant when different populations of animals are used. The estimates given in the Pharmacopoeia for the error of the assays of some of the antitoxins were obtained in this way, i.e. the same preparation was assayed in a number of different laboratories and the error calculated from the results.

For most methods of assay estimates of potency will be obtained on a logarithmic scale. In any case the analysis should be done on the logarithms of the potency estimates (M) as these should be normally distributed.

The variability of the different values of M, from one assay to another is conveniently measured by their standard deviation (s_M). This may be obtained by calculating the variance (s_M^2) as follows.

$$S_M^{\,2} = \frac{1}{n'-1}\sum(M-\overline{M})^2 = \frac{1}{n'-1}\left[\sum M^2 - \frac{\left(\sum M\right)^2}{n'}\right] \quad \ldots (1)$$

The second formula being the more suitable for desk calculation. Here $\overline{M}$ is the mean of the values of M, and n′ is the number of assays. The standard deviation, s_M, is then obtained as the square root of s_M^2.

Variances estimated by Equation 1 are unreliable when only a few assays of the same preparation have been carried out and large experiments, which would give more reliable estimates of variance, are rarely undertaken. Therefore it is generally more satisfactory to estimate a common value for the variance from results for several preparations each of which has been assayed two or more times. A pooled estimate of variance is then calculated as

$$s_M^{\,2} = \frac{\sum_P\left[M-\left(\overline{M}\right)^2\right]}{\sum_P(n'-1)} = \frac{\sum_P\left[\sum M^2-\left(\sum M\right)^2/n'\right]}{\sum_P(n'-1)} \quad \ldots (2)$$

where, $\sum_P$ denotes summation over the several preparations.

Equation 2 should not be used if there is evidence that variances are substantially different for different preparations.

The precision of a single potency estimate (R) may be expressed in terms of 0.95 fiducial (or confidence) limits, by the formula

$$\text{antilog}\,(M \pm ts_M) \quad \ldots (3)$$

The appropriate value of t may be obtained from Table 1 according to the number of degrees of freedom (f) given by the denominator of Equation 1 or 2.

It is expected that, 95 times out of 100, limits calculated in this way will contain the true potency.

Table 1 – Both sided values of t (P=0.95)

Degrees of freedom (f)	Both sided values of t	Degrees of freedom (f)	Both sided values of t
1	12.71	14	2.14
2	4.30	15	2.13
3	3.18	16	2.12
4	2.78	17	2.11
5	2.57	18	2.10
6	2.45	19-20	2.09
7	2.36	21	2.08
8	2.31	22-23	2.07
9	2.26	24-26	2.06
10	2.23	27-29	2.05
11	2.20	30-32	2.04
12	2.18	Infinity	1.96
13	2.16		

The fiducial limits for the potency, expressed as a percentage of the estimated potency, are the antilogarithms of ($2 \pm ts_M$). Example 1 illustrates the calculation for several assays of one preparation by use of Equation 1. Example 2 illustrates the calculation for more than one preparation by use of Equation 2. When the same preparation has been assayed n time, a single estimate of its activity may be obtained from the antilog of the mean ($\overline{M}$) of the various values of M.

Fiducial limits for the mean potency may be obtained as

$$\text{antilog}\left(\overline{M} \pm ts_{\overline{M}}\right) \quad \ldots (4)$$

where, $s_{\overline{M}}^2 = \frac{s_M^2}{n'}$ .... (5)

and s_M^2 is obtained from equation (1) or (2) as appropriate.

Example 1 – Estimate of error from repeated assays.

Potency estimate (R)	log R = M	M^2
0.738	$\bar{1}.8681 = -0.1319$	0.017398
0.766	$\bar{1}.8842 = -0.1158$	0.013410
0.803	$\bar{1}.9047 = -0.0953$	0.009082
0.817	$\bar{1}.9122 = -0.0878$	0.007709
0.870	$\bar{1}.9395 = -0.0605$	0.003660
0.889	$\bar{1}.9489 = -0.0511$	0.002611
Total	−0.5424	0.053870

The variance of M is. calculated from Equation 1.

$$S_M^2 = \frac{1}{5}\left[(0.053870) - \frac{(-0.5424)^2}{6}\right] = 0.000967$$

So that $S_M = 0.0311$

At P = 0.95 with 5 degrees of freedom, t = 2.57 (Table 1) or at 95 per cent fiducial levels with 5 degrees of freedom.

Fiducial limits as percentage of potency estimate

= antilog [2 ± (2.57)(0.0311)]

= antilog 1.9201 and antilog 2.0799

= 83.2 per cent to 120.2 per cent

These fiducial limits apply to any single estimate of potency from an individual assay.

Example 2 – Estimate of error from repeated assays with several preparations

Potency estimate (R)	Log R = M	M^2
Preparation I		
0.792	$\bar{1}.8987 = -0.1013$	0.010262
0.800	$\bar{1}.9031 = -0.0969$	0.009390
0.828	$\bar{1}.9180 = -0.0820$	0.006724
Total	−0.2802	0.026376
Preparation II		
0.852	$\bar{1}.9304 = -0.0696$	0.004844
0.905	$\bar{1}.9566 = -0.0434$	0.001884
Total	−0.1130	0.006728

The variances of M calculated from Equation 2

$$S_M^2 = \frac{1}{3}\left[\left\{(0.026376) - \frac{(-0.2802)^2}{3}\right\} + \left\{(0.006728) - \frac{(-0.1130)^2}{2}\right\}\right]$$

$$= \frac{1}{3}(0.000549) = 0.000183$$

So that $S_M = 0.0135$

At p = 0.05 with 3 degrees of freedom, t = 3.18 (Table 1) or at 95 per cent fiducial levels with 5 degrees of freedom.

Fiducial limits as percentage of potency estimate

= antilog [2 ± (3.18)(0.0135)]

= antilog 1.9571 and antilog 2.0429

= 90.6 per cent to 110.4 per cent

These confidential limits apply to any single estimate of potency from an individual assay.

5.7.6. Direct Assays along with Illustrative Example

In this type of assay, the doses which produce some fixed effect in each animal (e.g. death) are measured. The assay of a digitalis preparation using guinea – pigs is an example (Example 3) of such a test. There are generally two groups of estimates of individual effective doses, one showing the results with a solution of the standard, and the other the results with a solution of the preparation of unknown activity. The solutions are made up to be of approximately equal potency by assuming a potency for the unknown (A_U). Each result is converted to a logarithm, and the means of the log effective doses for the standard ($\bar{X}_S$) and the unknown ($\bar{X}_U$)are calculated. The potency ratio of unknown to standard before adjustment by the assumed potency (R') is the antilog of M'

where

$$M' = \bar{X}_S - \bar{X}_U \quad \ldots(6)$$

The variance of M' is the sum of the variances of the two means and is calculated from equation 7.

$$S_x^2 = S_x^2 \left[\frac{1}{N_S} + \frac{1}{N_u}\right] \quad \ldots(7)$$

$$S_x^2 = \frac{\left[\sum_s X_S^2 - \frac{(\sum X_S)^2}{N_S}\right] + \left[\sum_u X_u^2 - \frac{(\sum X_u)^2}{N_u}\right]}{N_S + N_U - 2} \quad \ldots(8)$$

N_S and N_U are the numbers of animals treated with standard and unknown and $\sum_S$ and $\sum_U$ represent the summation of results obtained with the two preparations. Fiducial limits are calculated as

$$\text{antilog}\,(M' \pm ts_{M'}) \quad \ldots(9)$$

The degrees of freedom for t are equal to $(N_S + N_U - 2)$. The estimate of potency (R) is equal to antilog (M) where

$$M = M' + \log A_U \quad \ldots(10)$$

with fiducial limits

$$\text{antilog}\,(M \pm t^* s_M) \quad \ldots(11)$$

In this way s_M is equal to $s_{M'}$. Limits calculated by Equation 11 may be expressed as percentage of the stated potency or as percentages of the estimated potency.

In a valid assay, the variance of X_S should be the same as that for X_U, apart from sampling errors. If necessary this condition may be tested by calculating the variances and dividing the larger by the smaller, to obtain a variance ratio.

The variance of X_S is calculated from Equation 12

$$S_{X_S}^2 = \frac{\sum_s X_s^2 - \frac{\left(\sum_s X_s\right)^2}{N_s}}{N_s - 1} \quad \ldots(12)$$

and similarly for $S_{X_u}^2$

The variance ratio (F) distribution is given in Table 6 but it should be noted that for this test the upper values given correspond to P = 0.05, and the lower values to P = 0.01.

Example 3 – Direct assay with digitalis using guinea-pigs

Preparation	Estimate of individual lethal dose (ml/kg)	X = log lethal dose
Standard Preparation (S)	1.12	0.0492
	1.44	0.1584
	1.06	0.0253
	1.14	0.0569
	1.26	0.1004
	1.20	0.0792
Total $\sum_s X_s$		0.4694
Mean $\bar{X}_S$		$\frac{1}{6}(0.4694)$ = 0.0782
Test preparation (U)	1.24	0.0934
	1.38	0.1399
	1.08	0.0334
	1.41	0.1492
	1.20	0.0792
	1.09	0.0374
Total $\sum_u X_u$		0.5325
Mean $\bar{X}_u$		$\frac{1}{6}(0.5325)$ = 0.0888

The variance S_x^2 is calculated as

$$S_x^2 = \frac{1}{10}\left[\left\{(0.0492)^2 + \ldots + (0.0792)^2 - \frac{(0.4694)^2}{6}\right\} + \left\{(0.0934)^2 + \ldots + (0.0374)^2 - \frac{(0.5325)^2}{6}\right\}\right]$$

= 002310

From Equation 7, $S^2_{M'} = 0.002310\left(\frac{1}{6}+\frac{1}{6}\right) = 0.000770$

So that $s_{M'} = 0.0277$

At P = 0.05 with 10 degrees of freedom, t = 2.23 (Table 1)

$M' = 0.0782 - 0.0888 = -0.0106 = \bar{1}.9894$

If the concentrations of the two solutions are equal, as in this example, then the assumed potency ratio equals unity and M = M'. Potency ratio (Test/Standard) = 0.98.

Fiducial limits to potency ratio

= antilog [- 0.0106 ± (2.23) (0.0277)]

= antilog 1.9276 and antilog 0.0512 = 0.85 to 1.13

If the test concentration (mg/ml) had been twice that of the standard, then the assumed potency ratio A would be 0.5. M would then be equal to M' + log 0.5.

5.7.7. Assays depending upon Measured Effects.

5.7.7.1. Validity and other conditions

These tests involve the measurement of the effects of fixed quantities of drugs on individual biological systems, e.g. a whole animal, isolated animal tissue or a culture of bacteria.

Each treatment which consists of a fixed dose of a standard (s_1, s_2) or of an unknown (u_1, u_2) to be assayed, is administered to a certain number (n) of experimental units (animals, cultures, tubes, etc.) and n responses are recorded, one per unit. The responses (y) may be increases in the weights of animals, organ weights, the sizes of zones of inhibition of bacterial growth, measurements of the turbidity of cultures, etc.

The methods of calculation described below may be used to evaluate the results of such assays provided that the following conditions are fulfilled.

1. The experimental units have been randomly assigned to the different treatments (Sections 5.7.4 and 5.7.7.2)
2. The responses to each treatment are normally distributed.
3. The standard deviation of the response is independent of the level of response, i.e. is constant for each treatment.
4. The relationship between the logarithm of the dose and the response can be represented by a straight line, over the range of doses used.
5. For any test preparation in the assay, the straight line (defined in 4) must be parallel to that for the standard.

If conditions 3 and 4 are not met, it may be possible to remedy the situation by using a different response metameter, i.e by transforming the response, e.g. by taking its square or logarithm before proceeding with the calculation.

It may be possible to assume conditions 2, 3 and 4 from preliminary studies of the method. Condition 4 (linearity) can only be verified in assays where at least three dilutions of each preparation have been tested and monographs which specify the use of only two dilutions assume that linearity has been established in previous studies of the log dose – response curve. Condition 5 (parallelism) should be tested in every assay, i.e. there should never be less than two dilution of each preparation. It is also desirable that test preparations should be assayed at doses which give responses approximately equal to those obtained with the corresponding doses of the standard preparation.

If any of the five conditions is not met, the methods of calculation described here are unreliable and a special study of the assay by an expert statistician will be required in order to decide what conclusions may be drawn from it.

When validity is established the potency of each unknown relative to that of the standard may be calculated and expressed as a potency ratio or converted to some unit appropriate to the preparation under examination, e.g. International Unit. Fiducial limits may also be estimated from each set of assay data.

In order to simplify the statistical analysis presented here it is necessary to impose the following restrictions on the assay design.

(a) Each preparation in the assay must be tested at the same number of dilutions. Formulae are given for assays using two dose levels.

(b) The ratio of adjacent doses must be constant for all treatments in the assay.

(c) There are an equal number of responses to each treatment.

If one response is missing, it can be estimated by methods given in Section 5.7.7.6

5.7.7.2. Assay Design

The allocation of individuals to different treatments may be made in various ways.

Random Design

If the totality of experimental units (animals, tubes, etc.) appears to be reasonably homogeneous, with no indication that variability in response will be smaller within certain recognizable sub-groups, the allocation of the units to the different treatments should be made at random, e.g. by using a table of random numbers.

If sub-groups such as litters, physical positions or experimental days are likely to be more homogenous than the totality of units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful choice of balance over these restrictions permits irrelevant sources of variation to be eliminated.

Randomised Block

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment is applied once in every block (litter or Petri dish) and is only suitable when the block is large enough to accommodate all treatments.

Cross-over Test

This design is useful when the experiment can be subdivided into blocks but it is possible to apply only two treatments to each block, e.g. a block may be a single animal which can be tested on two occasions. The design is intended to increase precision by eliminating the effects of differences between animals while balancing the effect of any difference between general levels of response at the two stages of the test. If two doses of a standard and of an unknown preparation are tested this is known as a twin cross-over test.

The experiment is divided into two parts separated by a suitable time interval. Animals are divided into 4 groups and each group receives one of the 4 treatments in the first part of the test. Animals which received one preparation in the first part of the test receive the other preparation on the second occasion and animals receiving small doses in one part of the test receive large doses in the other. The arrangement of doses is shown in Table 2.

Table 2 – Arrangement of doses in twin cross-over test

Group of animals	Day I	Day II
1	s_1	u_2
2	s_2	u_1
3	u_1	s_2
4	u_2	s_2

Whichever design is used, the allocation of experimental units to blocks should be made at random and the units should be kept under uniform conditions both before and during the experiment.

5.7.7.3. Analysis of Variance

Apart from some adjustments to the error term the basic analysis of data derived from an assay is the same for random and randomized block designs. This section gives formulae required to carry out the analysis and will be more easily understood by reference to the worked examples in section 5.7.8. Reference should also be made to the glossary of symbols (Section 5.7.3.). The formulae are appropriate for simple assays where a single unknown preparation (U) is compared with a standard preparation (S) and for multiple assays where several unknown (U……Z) are included. The formulae for cross-over tests do not entirely fit the scheme and these are incorporated into Example. 5.

Table 3 - Formulae for assays with two does of each preparation

Dose and response	Standard (S)	Ist test preparation (U)	(h-1)st test preparation (Z)
Low dose (total response)	S_1	U_1	Z_1
High dose (total response)	S_2	U_2	Z_2
For preparation (total response)	$S_1+S_2=S$	$U_1+U_2=U$	$Z_1+Z_2=Z$
Linear contrast	$S_2+S_1=L_S$	$U_2+U_1=L_U$	$Z_2+Z_1=L_Z$

Having considered the points discussed in Section 5.7.7.1 and transformed the responses (y) if necessary the values of y should be assumed over treatment and each preparation, as shown in table 3. The linear contrasts, which relate to the slopes of the log dose – response lines, should also be derived. The total variation in responses, caused by the different treatments, is now partitioned as shown in table 4, the sums of squares being derived from the values obtained in Table 2. K represents the square of the total of all responses recorded for the assay divided by the total number of responses.

Table 4 – Tests of validity for two-dose assays

Source of variation	Degrees of freedom (f)	Reduced sum of squares
Preparations	$h-1$	$\frac{(S^2+U^2+\ldots+Z^2)}{2n}-K$
Regression	1	$\frac{(L_s+L_u+\ldots+L_z)^2}{2nh}=E$
Parallelism	$h-1$	$\frac{(L_s{}^2+L_u{}^2+\ldots+L_z{}^2)}{2n}-E$

The residual error of the assay is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 5). In this table Σy^2 represents the sum of squares for all responses recorded in the assay. It should be noted that the reduced sum of squares for treatments is equal to the reduced sums of square in Table 4 totalled over all sources of variation.

5.7.7.4. Tests of Validity

In order to assess the significance of the sources of variation listed in Table 4 each of the reduced sums of squares, obtained from that table, should be divided by the corresponding

Table 5 – Estimation of residual error

Source of variation	Degrees of freedom (f)	Reduced sum of squares	
		Random design	Randomised block
Treatments	k – 1	$\frac{\left(S_1^2+S_2^2+...+Z_k^2\right)}{n}-K$	$\frac{\left(S_1^2+S_2^2+...+Z_k^2\right)}{hd}-K$
Blocks (rows)	n-1	—	$\frac{\left(R_1^2+R_2^2+...+R_n^2\right)}{hd}-K$
Residual error	By subtraction	*	*
Total	N – 1	Σy^2-K	Σy^2-K

*Obtained by subtracting from the total reduced sum of squares all other reduced sums of squares calculated for the particular design.

number of degrees of freedom to give mean squares. The mean square for residual error (s^2) is a similar quotient derived from the appropriate line in Table 5.

The mean square for each variable to be tested is now expressed as a ratio to s^2 and the significance of these values (known as F ratios) assessed by use of Table 6. Critical values of F, for a chance occurrence of 5 per cent or 1 per cent, may be found by reference to the column of the Table corresponding to the number of degrees of freedom associated with the mean square for the variable being tested (f_1) and the row of the Table corresponding to the number of degrees of freedom associated with s^2 (f_2). If a calculated F value is larger than the tabulated value, the variable being tested is said to be *'significant'* at the level of probability indicated, i.e. 0.05 or 0.01.

Assay results are said to be *'statistically valid'* if the outcome of these tests is as follows.

1. The regression term should be highly significant, i.e. the calculated F should be larger than the tabulated F for P = 0.01. This indicates that the slope of the log dose–response line is satisfactory.
2. The parallelism term should not be significant (see condition 5, section 5.7.7.1)

When statistical validity is established potencies and fiducial limits may be estimated by methods described in the next section.

5.7.7.5. Estimation of Potency and Fiducial Limits

The mean response for each preparation $\left(\bar{y}_s, \bar{y}_u ... \bar{y}_z\right)$ should first be calculated.

$$\bar{y}_s = \frac{S}{N_S} \qquad \text{.... (13)}$$

and similarly for the other preparations.

If I is the interval between adjacent log doses of any preparation, the common slope (b) for assays with two doses of each preparation is obtained from equation 14.

$$b = \frac{1}{Inh}\left(L_s + L_u + ... + L_z\right) \qquad \text{.... (14)}$$

The logarithm of the potency ratio of a test preparation U(M'_U) is

$$\frac{\bar{y}_u - \bar{y}_s}{b} \qquad \text{.... (15)}$$

The calculated potency is an estimate of the true potency of each unknown. Fiducial limits (which have a 95 per cent probability of including the true potency) may be calculated as the antilogarithms of Formula 16.

$$CM'_u \pm \frac{st\sqrt{C}}{b}\sqrt{\frac{1}{N_s}+\frac{1}{N_u}+\frac{\left(\bar{y}_s-\bar{y}_u\right)^2}{E-s^2t^2}} \qquad \text{.... (16)}$$

where, $C = \frac{E}{E-s^2t^2}$ .... (17)

E is obtained for Table 4, s^2 is the residual error from Table 5 divided by its degrees of freedom and t is read from Table 1 according to degrees of freedom for s^2.

For the balanced two dose assays described here the formula (16) for limits can be simplified to:

$$CM'_u \pm \sqrt{(C-1)\left(CM'^2_u + c'I^2\right)} \qquad \text{.... (18)}$$

where c′ is a coefficient obtained from Table 7.

Table 6 – The variance ratio (F) distribution

Degrees of freedom for denominator (f_2)	Degrees of freedom for numerator (f_1)									
	1	2	3	4	5	6	7	8	20	Infinity
12	**4.75**	**3.89**	**3.49**	**3.26**	**3.11**	**3.00**	**2.91**	**2.85**	**2.54**	**2.30**
	9.33	6.93	5.95	5.41	5.06	4.82	4.65	4.50	3.86	3.36
15	**4.54**	**3.68**	**3.29**	**3.06**	**2.90**	**2.79**	**2.71**	**2.64**	**2.33**	**2.07**
	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.37	2.87
20	**4.35**	**3.49**	**3.10**	**2.87**	**2.71**	**2.60**	**2.51**	**2.45**	**2.12**	**1.84**
	8.10	5.85	4.94	4.43	4.10	3.87	3.70	3.56	2.94	2.42
30	**4.17**	**3.32**	**2.92**	**2.69**	**2.53**	**2.42**	**2.33**	**2.27**	**1.93**	**1.62**
	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	2.55	2.01
60	**4.00**	**3.15**	**2.76**	**2.53**	**2.37**	**2.25**	**2.17**	**2.10**	**1.75**	**1.39**
	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.20	1.60
Infinity	**3.84**	**3.00**	**2.60**	**2.37**	**2.21**	**2.10**	**2.01**	**1.94**	**1.57**	**1.00**
	6.63	4.61	3.78	3.32	3.02	2.80	2.64	2.51	1.88	1.00

The upper bold values correspond to P = 0.05, the lower values to P = 0.01.

C is a measure of the significance of the regression and in an assay with a well-defined slope the value of C will be very close to unity.

Table 7 – Constant used in formula for fiducial limits: with two doses of each preparation values of c' are given below.

Number of test Preparations (h – 1)	1	2	3	4	5
Values of c'	1	3/2	2	5/2	3

The potency ratio (R_U) and associated fiducial limits are obtained either by multiplying the values obtained from Formulae 15 and 18 by A_U after antilogarithms have been taken or by adding log A_U being taking antilogarithms.

5.7.7.6. Missing Values

In a balanced assay, an accident unconnected with the applied treatments may lead to the loss of one or more responses, for example, because an animal dies. Full statistical analysis is then much more complicated. However, if only one value is missing, an approximate analysis can keep the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom, for the total sum of squares and for the residual error, by unity, and using one of the following equations for the missing value.

Random Design

In a completely randomized assay the missing value can be replaced by the arithmetic mean of the other responses to the same treatment.

Randomised block

The missing value (y') is obtained by use of Equation 19.

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)} \quad \ldots (18)$$

Where B' is the sum of responses in the block containing the missing value, T' the corresponding treatment total and G' is the sum of all responded in the assay.

Example: Suppose that the response to dose u_1 in the first block of the heparin assay (Example 4 : Section 5.7.8.1) was missing.

$$B' = 12.440, T' = 7.122, G' 56.926, y' = 2.371$$

The value 2.371 would appear in the table of responses in place of 2.352 and calculation would proceed as in Example 4 but the degrees of freedom for 'error' would be 14 and for '*total*' they would be 22.

Cross-over Design

If an accident leading to loss of values occurs in a twin cross-over design, consultation with a statistician is essential,

Table 8 – Response metameter y (logarithm of coagulation time in seconds)

Block	Standard S		Test U		Test Z		Block total
	s_1	s_2	u_1	u_2	z_1	z_2	
1	2.348	2.591	2.352	2.588	2.335	2.578	R_1 =14.792
2	2.371	2.571	2.365	2.582	2.352	2.568	R_2 =14.809
3	2.342	2.580	2.380	2.601	2.339	2.565	R_3 =14.807
4	2.358	2.594	2.377	2.618	2.346	2.577	R_4 =14.870

Table 9 – Response totals and contrasts

Dose and response	Preparation			
	Standard S	Test U	Test Z	Total
Low dose	S_1 = 9.419	U_1 = 9.474	Z_1 = 9.372	
High dose	S_2 = 10.336	U_2 = 10.389	Z_2 = 10.288	
Preparation totals	S = 19.755	U = 19.863	Z = 19.660	Σy = 59.278
Linear contrasts	L_S = 0.917	L_U = 0.915	L_Z = 0.916	ΣL = 2.748

Table 10 – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean Square	F	P
Preparations	2	0.00258	0.00129		
Regression	1	0.31465	0.31465	2689	< 0.01
Parallelism	2	0.00000	0.00000	0	> 0.05
Treatments	5	0.31723	0.06345		
Blocks	3	0.00060	0.00020		
Error	15	0.00176	0.000117		
Total	23	0.31959			

because the appropriate equations depend upon the particular treatment combinations.

Similarly, consult a statistician if more than one responses are missing in any of the designs.

5.7.8. Examples of Assays Depending upon Measured Effects

This section consists of worked examples illustrating the application of formulae described in section 5.7.7.

Formulae for a two- dose multiple assay arranged in randomized block design are used in Example 4.

Some extra notation has been introduced in the example of a cross-over assay (Example 5) for, in order to carry out analysis, it is necessary to form treatment totals and linear contrasts for each day of the test, separately AI or II added to the subscript of a symbol indicates that the value relates to the first or second occasion of testing. D_I and D_{II} are the response totals for days I and II and $B_1, B_2 \ldots\ldots B_{2n}$ are the sums of the paired responses for each experimental unit (i.e. animal)

5.7.8.1. Example 4 : Two – Dose Multiple Assay, Randomized Block Design

Assay of heparin by consideration of blood clotting

The standard preparation contains 130 units per mg. Doses of the standard used in the assay were 1.4 and 2.0 units per ml. Both test preparations were assigned nominal potencies of 130 units per mg and doses were prepared equivalent to those of the standard. Table 8 summarizes the data.

$$\text{Correction term } K = \frac{(\Sigma y)^2}{N} = \frac{(59.278)^2}{24} = 146.41172$$

Sum of squares for blocks, using the formula in Table 5, is given by

$$\frac{\left(R_1^2+R_2^2+R_3^2+R_4^2\right)}{6}-K=0.00060$$

The other sums of squares (see Table 9) are obtained in the same manner.

Validity of assay

The analysis of variance satisfactorily confirmed significant regression between dose levels and with a sum of squared for deviation from parallelism being equal to zero there was no question of the departure from parallelism of the regression lines being significant.

Calculation of potency ration and fiducial limits

Doses administered were 1.4 and 2.0 units per ml;

$I=\log 2.0-\log 1.4=0.1549$

$t=2.13$ from Table 1 with 15 degrees of freedom.

$$b=\frac{\Sigma L}{\text{Inh}(d-1)}=\frac{2.748}{[(0.1549)\{(4\times3)\times(2-1)\}]}=1.4784$$

$\bar{y}_s=19.755/8=2.4694,\ \bar{y}_u=2.4829,\ \bar{y}_Z=2.4575$

$M'_u=(\bar{Y}_u-\bar{Y}_s)/b=0.00913, M_u=M'_u$ since $\log A_U=0$

Potency ratio for test U = antilog M_u = 1.02.

$C=E/(E-s^2t^2)=0.31465/[0.31465-(0.000117)(2.13)^2]$

$=1.00170$

$c'=3/2$ from Table 7.

Fiducial limits potency ration given by

$$A_u \text{antilog}\left[CM'_u \pm \sqrt{(C-1)(CM'^2_u+C'I^2)}\right]$$

$$=\text{anti log}\left[(0.009146)\pm\sqrt{(0.0017)(0.0000835+0.0359910)}\right]$$

$=\text{antilog}[(0.009146)\pm(0.007831)]$

= antilog (0.0013) and antilog (0.0170)

Fiducial limits to potency ratio of test U are 1.00 to 1.04. Using the same procedure, potency ratio for test Z is 0.98 with fiducial limits 0.96 to 1.00

5.7.8.2. Example 5 : Twin Cross–Over Assay

Assay of insulin using rabbits

Standard doses used were 1 and 2 units per ml. Equivalent doses of the test solution were prepared based on an assumed potency of 40 units per ml. The rabbits were injected subcutaneously with 0.5 ml of the appropriate solutions, according to the design in Table 11.

Table 11 – Arrangements of treatments

Day	Group of rabbits			
	1	2	3	4
I	S_1	S_2	U_1	U_2
II	U_2	U_1	S_2	S_1

The analysis of variance is more complicated for this assay than for the other designs given because the component of the sum of squares due to parallelism is not independent of the component due to rabbit differences.

Testing of the parallelism of the regression lines involves a second error mean square term obtained by subtracting the parallelism component and two '*interaction*' components from the component due to rabbit differences.

Table 12 – Response y (sum of blood glucose readings (mg per cent) at 1½ hours)

Group 1			Group 2			Group 3			Group 4		
s_1	u_2	Total	s_2	u_1	Total	u_1	s_2	Total	u_1	s_1	Total
112	104	216	65	72	137	105	91	196	118	114	262
126	112	238	116	160	276	83	67	150	119	149	268
62	58	120	73	72	145	125	67	192	42	51	93
86	63	149	47	93	140	56	45	101	64	107	171
52	53	105	88	113	201	92	84	176	93	117	210
110	113	223	63	71	134	101	56	157	73	128	201
116	91	207	50	65	115	66	55	121	39	87	126
101	68	169	55	100	155	91	68	159	31	71	102

Three *'interaction'* components are present in the analysis of variances due to replication within each group:

days × preparations, days × regression, days × parallelism

These terms indicate the tendency for the components (preparation, regression and parallelism) to vary from day to day. The corresponding F tests thus provide checks on these aspects of assay validity. If the values of F obtained are significantly high, care should be exercised in interpreting the results of the assay, and if possible the assay should be repeated.

The values for sums of squares were obtained using the quantities in Tables 12 and 13.

Table 13 – Response totals and contrasts

Dose	Test		
	Standard S	Test U	Total
Day I			
Low dose	$S_{II} = 765$	$U_{II} = 719$	
High dose	$S_{2I} = 557$	$U_{2I} = 579$	
Total	$S_I = 1322$	$U_I = 1298$	$D_I = 2620$
Day II			
Low dose	$S_{III} = 854$	$U_{III} = 746$	
High dose	$S_{2II} = 533$	$U_{2II} = 662$	
Total	$S_{II} = 1387$	$U_{II} = 1408$	$D_{II} = 2795$
Preparation total	$S = 2709$	$U = 2706$	$\Sigma y = 5415$
Linear contrast			
Day I	$L_{SI} = -208$	$L_{UI} = -140$	$L_I = -348$
Day I	$L_{SII} = -321$	$L_{UII} = -84$	$L_{II} = -405$
Total	$L_S = -529$	$L_U = -224$	$\Sigma L = -753$

$$\text{Correction term } K = \Sigma(y)^2/N = (5415)^2/64 = 458160$$

$$\text{Total} = (\Sigma y)^2 - K = 511583 - 458160 = 53423$$

$$\text{Blocks} = \frac{B_1^{\,2} + B_2^{\,2} + B_{2n}^{\,2}}{2} - K$$

$$= \frac{(216)^2 + (238)^2 + \ldots + (102)^2}{2} - 458160 = 39795$$

$$\text{Preparations} = \frac{S^2 + U^2}{2n} - K$$

$$= \frac{(2709)^2 + (2706)^2}{32} - 458160 = 0$$

$$\text{Days} = \frac{D_I^{\,2} + D_{II}^{\,2}}{2n} - K = \frac{(2620)^2 + (2795)^2}{32} - 458160 = 478$$

$$\text{Regression} = \frac{(L_s + L_u)^2}{N} = \frac{(-753)^2}{64} = 8860 = E$$

$$\text{Parallelism} = \frac{\left(L_s^{\,2} + L_u^{\,2}\right)}{2n} - E$$

$$= \frac{(529)^2 + (224)^2}{32} - 8860 = 1453$$

$$\text{Days} \times \text{Regression} = \frac{L_I^{\,2} + L_{II}^{\,2}}{2n} - E$$

$$= \frac{(348)^2 + (405)^2}{32} - 8860 = 50$$

$$\text{Days} \times \text{Parallelism} = \frac{L_{SI}^{\,2} + L_{SII}^{\,2} + L_{UI}^{\,2} + L_{UII}^{\,2}}{n} - E$$

$$- \text{Parallelism} - \text{days} \times \text{Regression}$$

$$= \frac{(208)^2 + (321)^2 + (140)^2 + (84)^2}{16} - 8860 - 1453 - 50 = 447$$

$$\text{Days} \times \text{Preparations} = \frac{S_I^{\,2} + S_{SII}^{\,2} + U_I^{\,2} + U_{II}^{\,2}}{n} - K$$

$$- \text{days} - \text{Preparations}$$

$$= \frac{(1322)^2 + (1387)^2 + (1298)^2 + (1408)^2}{16} - 458160 - 478$$

$$= 32$$

$$\text{Error (1)} = \text{Blocks} - \text{Parallelism} - (\text{Days} \times \text{Preparation})$$

$$- (\text{Days} \times \text{Regression}) = 38260$$

$$\text{Error (II)} = \text{Total} - \text{Blocks} - \text{Preparation} - \text{Regression}$$

$$- \text{Days} - (\text{Days} \times \text{Parallelism}) = 3843$$

Validity of assay

The analysis of variances confirmed that the data fulfilled the necessary conditions for a satisfactory assay.

(i) Significant regression. The F – value 64.5 for regression, calculated using the mean square of error (II), is higher than the interpolated critical value given in Table 6 for P = 0.01, $f_1 = 1$, $f_2 = 28$.

(ii) Departure from parallelism of the regression lines. The test for parallelism in a cross-over assay is not very sensitive, being based on the mean square of error (I) which depends on variation between the rabbits used. The F- value of 1.06 is less tan the interpolated critical value in Table 6 for P = 0.05, $f_1 = 1$, $f_2 = 28$

Table 14 – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F	P
Parallelism	1	1453	1453	1.06	>005
Days × preparations	1	32	32	0.02	>0.05
Days × regression	1	50	50	0.04	>0.05
Error (I)	28	38260	1366		
Blocks (rabbits)	31	39795	1284	9.35	
Preparations	1	0	0	0.00	>0.05
Regression	1	8860	8860	64.5	<0.01
Days	1	478	478	3.48	>0.05
Days × parallelism	1	447	447	3.26	>0.05
Error (II)	28	3843	137.3		
Total	63	53423			

N = 64, total number of responses ; n = 16, number of replicated per dose.

(iii) None of the three interaction components was significant, the three F – values being 0.02, 0.04 and 3.26.

Calculation of potency estimate and fiducial limits

I = log 2.0 – log 1.0 = 0.3010, t = 2.05 with 28 degrees of freedom from Table 1. For a twin cross – over design, we have

$$b = \frac{2(L_s + L_u)}{NI} = \frac{2(-529 - 224)}{64X0.0310} = -78.17,$$

$$\bar{y}_S = \frac{S}{2n} = \frac{2709}{32} = 84.66, \bar{y}_u = 84.56$$

$$M'_u = (\bar{y}_u - \bar{y}_s)/b = 0.0013$$

A_U = 40 Units per ml, $M_u = M'_u + \log A_u = 1.6034$

$C = E/(E - s^2t^2) = 8860/[8860 - 137.3(2.05)^2] = 1.0697$

c′ = 1 from Table 7.

log fiducial limits to potency of test U are given by

$$CM'_u \pm \sqrt{(C-1)\left(CM'^2_u + c'I^2\right)} + \log A_U$$

i.e. (1.0697×0.0013)

$$\pm\sqrt{(0.0697)[(1.0697)(0.0013)^2 + (0.03010)^2]} + 1.6021$$

$$= 0.0014 \pm \sqrt{0.00632} + 1.6021 = 1.6035 \pm 0.0795$$

Log fiducial limits = 1.5240 and 1.6830,

Fiducial limits to potency of test U are, therefore, 33.4 to 48.2 units per ml.

5.7.9. Assays depending upon Quantal effects

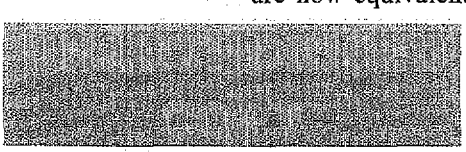
5.7.9.1. Introduction

In certain assays, such as that for insulin by subcutaneous injection into mice, it is impossible or excessively laborious to measure the effect on each animal on a quantitative scale. Instead, an effect such as death or hypoglycemic symptoms may be observed as either occurring or not occurring in each animal, and the result depends on the number of animals in which it occurs, such assays are called quantal or all-or-none. The situation is very similar to that described for quantitative assays, but in place of the n separate response to each treatment a single value is recorded, i.e. the percentage of animals in each treatment group showing a positive effect. When these percentages are plotted against the logarithms of the doses the resulting curve will tend to be sigmoid (S-shaped) rather than linear. A more satisfactory straight line relationship is obtained if a mathematical function of the percentage is used as response, in the statistical analysis of assay data, rather than the untransformed percentages. The most commonly used transformation is that of '*probit*'.

5.7.9.2. Modified Probit Method

The approximate method of analysis, described in this section, can be used provided that percentage of 0 or 100 do not occur in the data.

As shown in Example 6, the percentage of animals giving a positive response to each treatment is converted to a probit by use of table 15. For the purpose of calculation these probits are now equivalent to response values (y). A weighting



coefficient (w), corresponding to each probit, is obtained from table 16.

Formulae for the sums of squares, required in the analysis of variance, are the same as those used for quantitative assays (Table 3) with the exception of the error term (s^2) which should be calculated from Equation 20.

$$S^2 = \frac{k}{n\Sigma w} \quad \text{.... (20)}$$

Where k is the number of treatments, n is the number of animals tested is each treatment group and â w is the sum of the weighting coefficients.

The potency and fiducial limits are calculated by use of Formulae 15 and 18.

This approximate method will give results close to those obtained with the general probit method when percent responses for low and high doses are evenly spaced about 50 per cent. This implies that the weighting coefficients (w)

Table 15 – Probits corresponding to percentages

	0	1	2	3	4	5	6	7	8	9
0	–	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.00	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Table 16 – Weighting coefficients corresponding to probits

	Probits				
	1	2	3	4	
0.0	0.001	0.015	0.131	0.439	–
0.1	0.001	0.019	0.154	0.471	0.9
0.2	0.001	0.025	0.180	0.503	0.8
0.3	0.002	0.031	0.208	0.532	0.7
0.4	0.002	0.040	0.238	0.558	0.6
0.5	0.003	0.050	0.269	0.581	0.5
0.6	0.005	0.062	0.302	0.601	0.4
0.7	0.006	0.076	0.336	0.616	0.3
0.8	0.008	0.092	0.370	0.627	0.2
0.9	0.011	0.110	0.405	0.634	0.1
–	0.015	0.131	0.439	0.637	0.0
	8	7	6	5	
	Probits				

are approximately equal at each treatment level is assays with two doses of each preparation.

5.7.9.3. Example 6: Two-Dose Quantal Assay, Simplified Probit Method

Assay of insulin by subcutaneous injection in mice

Standard doses administered were 24 and 40 milliUnits per mouse, contained in a volume of 0.25 ml. Equivalent test doses used were prepared assuming the concentration of the test solution to be 40 units per ml.

A positive response was defined as a convulsion due to hypoglycaemia within 75 minutes of a subcutaneous injection of insulin (Table 17).

Table 17 – Positive responses from groups of 24 mice

Test / Response	Standard S		Test U	
	s_1	s_2	u_1	u_2
Number of positive response	8	21	10	20
Percentage response	33.3	87.5	41.7	83.3

$$\text{Correction term, } K = \frac{(S+U)^2}{k} = \frac{(21.48)^2}{4} = 115.3476$$

The values for sums of squares relating to preparations, regression and parallelism were obtained from the formulae in Table 5, taking n = 1.

$$\text{Preparation} = \frac{(S^2+U^2)}{2} - K$$

$$= \frac{\left[(10.73)^2+(10.75)^2\right]}{2} - 115.3478 = 0.0001$$

$$\text{Regression} = \frac{(L_s+L_u)^2}{4} = \frac{(2.76)^2}{4} = 1.9044 = E$$

$$\text{Parallelism} = \frac{(L_s^{\,2}+L_u^{\,2})}{2} - E$$

$$= \frac{\left[(1.59)^2+(1.17)^2\right]}{2} - 1.9044 = 0.0441$$

Error mean square is estimated by

$$\frac{K}{\sum nw} = \frac{4}{24(0.595+0.384+0.626+0.452)} = 0.0810$$

Table 19 – Analysis of variance

Source of variation	Degree of freedom	Sum of squares	Mean of square	F	P
Preparations	1	0.0001	0.0001		
Regression	1	1.9044	1.9044	23.51	<0.01
Parallelism	1	0.0441	0.0441	0.54	>0.05
Error	Infinite		0.0810		

Validity of assay

Significant regression between dose – levels and no suggestion of departure from parallelism of the individual regression lines indicate a satisfactory assay from which a potency estimate may be calculated.

Calculation of potency estimate and fidicual limits

I = log 40 – log 24 = 0.2219, t = 1.96 with infinite degrees of freedom (Table 1).

$$b = \frac{(L_s+L_u)}{Ih(d-1)} = \frac{2.76}{0.2219\times 2} = 6.2190$$

$$\bar{y}_s = \frac{S}{2} = 5.385; \bar{y}_u = \frac{U}{2} = 5.375$$

$$M'_u = \frac{(\bar{y}_u - \bar{y}_s)}{b} = \frac{(0.01)}{6.2190} = 0.0016$$

$M_u = M'_u + \log A_u = 0.0016 + 1.6021$

$R_u = 40.2$ Units per ml.

$C = E/(E - s^2t^2) = 1.9044/[19044 - (0.08100)(1.96)^2]$

$= 1.1953$

Table 18 – Probit transformation, response totals and contrasts

Test / Response	Standard S		Test U		Total
	s_1	s_2	u_1	u_2	
Probit response (Table 15)	$S_1 = 4.57$	$S_2 = 6.16$	$U_1 = 4.79$	$U_2 = 5.96$	
Weight w (Table 16)	0.595	0.384	0.626	0.452	
Preparation totals	$S = S_1 + S_2 = 10.73$		$U = U_1 + U_2 = 10.75$		$\sum y = 21.48$
Linear contrast	$L_s = S_2 - S_1 = 1.59$		$L_u = U_2 - U_1 = 1.17$		$\sum I = 2.76$

$c' = 1$ from Table 7.

Log fiducial limits to potency of test U are given by

$$CM'_u \pm \sqrt{(C-1)(CM'^2_u + c'I^2)} + \log A_U$$

giving fiducial limits of 32.1 to 50.4 Units per ml. Using the full method of probit analysis a potency estimate of 40.9 Units per ml was obtained with limits of 32.6 to 51.4 Units per ml.

5.7.10. Combination of Potency Estimates

5.7.10.1. Introduction

When the same preparation has been assayed several times it is often desirable to combine the resulting set of potencies into a single value, giving an overall potency that represents all the information available. There are several methods for combining the results of repeated assays, the most theoretically acceptable being the most difficult to apply.

A simple method of combination is described in Section 5.7.5, where the mean potency is derived from the antilogarithm of the arithmetic mean of the n' values of M and Formula 4 is used for the calculation of fiducial limits. This method has the disadvantage that assays with different numbers of response, or assays carried out with greater or less precision, will make equal contributions to the mean. A simple weighted method, which takes some account of these variations, is described in the following paragraphs.

In using the formulae from Section 5.7.5 or the ones in this section, two points should be taken care of

1. Estimates of log potency should be corrected by the assumed potency before they are combined.
2. The estimates should be independent, i.e. each should have been obtained from a separate assay which gave a set of responses to the standard preparation as well as to the unknown preparation under test.

5.7.10.2. Weighted Mean Potency And Fidicual Limits

It is assumed that the results of each of the n' assays have been analysed to given n' values of M with associated fiducial limits in logarithms, e.g. by use Formula 16.

For each assay the logarithmic fiducial interval (L) is obtained by subtracting the lower limit from the upper. A weight (W) for each value as that used in the calculation of fiducial limits, i.e. the value in Table 1 corresponding to degrees of freedom for the error mean square in the appropriate analysis of variance.

$$W = \frac{4t^2}{L^2} \qquad \text{.... (21)}$$

The products WM are formed for each assay and their sum divided by total weight for all assays to give the logarithm of the weighed mean potency $\overline{M}$, as shown in Equation 22.

$$\overline{M} = \frac{\sum_{n'} WM}{\sum_{n'} W} \qquad \text{.... (22)}$$

The standard error of the mean potency ($S_{\overline{M}}$) is taken to be the square root of the reciprocal of the total weight.

$$S_{\overline{M}} = \sqrt{1/\sum_{n'} W} \qquad \text{.... (23)}$$

and approximate confidential limits, which are expected to contain the true potency of the unknown preparation with 95 per cent probability, are obtained from the antilogarithms of the values given by Formula 24. The appropriate value of t in table 1 is that corresponding to the sum of the number of degrees of freedom for the error mean squares in the individual assays.

$$\overline{M} \pm t s_{\overline{M}} \qquad \text{.... (24)}$$

This approximate method of combination should give satisfactory results, provided that C is less than 1.1 for each of the n' assays and also that the individual potency estimates form a homogeneous set. A test for homogeneity is described in Section 5.7.10.3.

5.7.10.3. Homogeneity of Potency Estimates

The homogeneity of a set of log potency estimates may be tested by means of the statistic approximate chi-square (χ^2) (Table 20).

Table 20 – Value of χ^2 (P= 0.95)

Degrees of Freedom	χ^2 value
1	3.84
2	5.99
3	7.81
4	9.49
5	11.07
6	12.59
7	14.07
8	15.51
10	18.31
15	25.00
20	31.41
25	37.65

A value of χ^2 is calculated by squaring the deviation of each value of M from the weighted mean ($\overline{M}$), multiplying by the appropriate weight (W) and summing over all assays (Equation 25),

$$\chi^2 = \sum_{n'} W(M - \overline{M})^2 \quad \text{.... (25)}$$

If the calculated χ^2 is smaller than the tabulated value corresponding to $(n' - 1)$ degrees of freedom the potencies are homogeneous and the mean potency and limits obtained by the method of Section 5.7.10.2 will be meaningful.

If the calculated χ^2 is greater than the appropriate value in Table 20, potencies are heterogeneous. This means that the variation between individual estimates of M is greater than would have been predicated from the estimates of fiducial limits. Under these circumstance Formulae 22 and 24 are not applicable.

5.8. Dimensions of Capsule Shells

5.8.1. Dimensions of Hard Gelatin Capsule Shells

Hard Gelatin Capsule Shells normally used for the incorporation of medicaments are cylindrical in shape but other shapes are also formed for special requirements. The shells of the capsules consist of two prefabricated cylindrical sections, one end of which is rounded and the other is open. The shells are of various sizes, usually designated by different numbers, 5 being the smallest and 000 the largest. The dimensions of hard gelatin capsule shells tend to vary with the content of moisture in them and the conditions under which they are stored or to which they are exposed. The chemical composition of the shells also influence the extent to which exposure to heat and moisture affects the dimensions. Nevertheless, the average conventional dimensions (outside diameter, length and wall thicknes – single/double) of the capsule shells of sizes 000 to 5 are provided in the table 1, 2 and 3 for the guidance of users. It should be noted that any measurement of reasonable accuracy can be made only under controlled conditions of temperature and humidity. A temperature between 23° and 27° and a relative humidity between 45 per cent and 55 per cent are recommended.

Table 1 – Average Outside Diameter

Size	Cap (mm)	Body (mm)
000	9.89–10.01	9.54–9.66
00	8.49–8.61	8.16–8.28
0	7.60–7.72	7.28–7.40
1	6.87–6.99	6.57–6.69
2	6.31–6.43	6.02–6.14
3	5.77–5.89	5.50–5.62
4	5.27–5.39	5.01–5.13
5	4.85–4.97	4.59–4.71

Table 2 – Average Length

Size	Cap (mm)	Body (mm)
000	12.5–13.5	21.7–22.7
00	11.3–12.3	19.7–20.7
0	10.2–11.2	18.0–19.0
1	9.3–10.3	16.1–17.1
2	8.5–9.5	14.7–15.7
3	7.6–8.6	13.1–14.1
4	6.7–7.7	11.7–12.7
5	5.7–6.7	8.8–9.8

Table 3– AverageWall Thickness

Size	Cap (mm)	Body (mm)
000	0.095-0.125	0.095-0.125
00	0.095-0.125	0.090-0.120
0	0.095-0.125	0.090-0.120
1	0.090-0.120	0.087-0.117
2	0.090-0.120	0.087-0.117
3	0.085-0.115	0.082-0.112
4	0.082-0.112	0.082-0.112
5	0.082-0.112	0.082-0.112

NOTE — Multiply above values by 2 to arrive at norms for double wall thickness.

"Average for special dimensions may be decided upon mutually between the manufacturer of the Hard Gelatin Capsule Shells and the user."

5.8.2. Dimensions of Hard Cellulose Capsule Shells

Hard Cellulose Capsule Shells normally used for the incorporation of medicaments are cylindrical in shape but other shapes are also formed for special requirements. The shells of the capsules consist of two prefabricated cylindrical sections, one end of which is rounded and the other is open. The shells are of various sizes, usually designated by different numbers, 5 being the smallest and 000 the largest. The dimensions of hard cellulose capsule shells tend to vary with the content of moisture in them and the conditions under which they are stored or to which they are exposed. The chemical composition of the shells also influences the extent to which exposure to heat and moisture affects the dimensions. Nevertheless, the average conventional dimensions (outside diameter, length and wall thickness – single / double) of the capsule shells of sizes 000 to 5 are provided in the table 1, 2 & 3 for the guidance of users. It should be noted that any

measurement of reasonable accuracy can be made only under controlled conditions of temperature and humidity. A temperature between 23°and 27°and a relative humidity between 45 per cent and 55 per cent are recommended.

Table 1 – Average Outside Diameter

Size	Cap (mm)	Body (mm)
000	9.91 – 10.03	9.56 – 9.68
00	8.49 – 8.61	8.15 – 8.27
0	7.60 – 7.72	7.26 – 7.38
1	6.87 – 6.99	6.55 – 6.67
2	6.31 – 6.43	6.02 – 6.14
3	5.79 – 5.91	5.51 – 5.63
4	5.28 – 5.39	5.00 – 5.12
5	4.85 – 4.97	4.59 – 4.71

Table 2 – Average Length

Size	Cap (mm)	Body (mm)
000	12.5 – 13.5	21.7 – 22.7
00	11.3 – 12.3	19.7 – 20.7
0	10.2 – 11.2	18.0 – 19.0
1	9.3 – 10.3	16.1 – 17.1
2	8.5 – 9.5	14.7 – 15.7
3	7.6 – 8.6	13.1 – 14.1
4	6.7 – 7.7	11.7 – 12.7
5	5.7 – 6.7	8.8 – 9.8

Table 3 – Average Wall Thickness

Size	Cap (mm)	Body (mm)
000	0.095 - 0.125	0.095 - 0.125
00	0.095 - 0.125	0.095 - 0.125
0	0.095 - 0.125	0.095 - 0.125
1	0.090 - 0.120	0.090 - 0.120
2	0.090 - 0.120	0.090 - 0.120
3	0.085 - 0.115	0.085 - 0.115
4	0.082 - 0.112	0.082 - 0.112
5	0.082 - 0.112	0.082 - 0.112

NOTE — Multiply above values by 2 to arrive at norms for double wall thickness.

"Average for special dimensions may be decided upon mutually between the manufacturer of the Hard Gelatin Capsule Shells and the user."

5.9. Reference Substances

Reference Substances are highly characterized substances selected for their critical attributes and suitability for the intended purpose. They are specimens of drug substances, impurities, degradation products, herbal-related substances, biotechnology derived products, blood-related substances, vaccines, excipients, reference microbial cultures, reference spectra, and performance verification reference substances. Reference substances are specifically required in many Pharmacopoeial tests and assays to achieve adequate quality control of medicinal products and their components. They are never intended for use as drugs for therapeutic use.

TYPES OF REFERENCE SUBSTANCES

IP Reference Substances (IPRS)

Indian Pharmacopoeia Reference Substances abbreviated, as IPRS in the individual monographs, are issued by the Indian Pharmacopoeia Commission (IPC) or by laboratories authorized by the IPC and assume the official status in India. Where an IPRS is referred to in an official IP monograph, it becomes a part of the official standard that is alone authoritative in case of doubt or dispute to determine compliance with the IP.

IPRS are provided as pure materials or as mixtures of chemicals reflective of the corresponding drug substances or excipients and are generally employed in the identification and assay of articles as specified in IP monographs and general chapters. Official IPRS are primary standards that have the highest metrological quality within a specified context and are accepted without requiring comparison to another substance. IPRS are shown to be suitable for their intended purpose as specified in the IP monographs. The suitability of the IPRS for any purpose other than for which it has been established shall be demonstrated by the user. The property value or assay value of the IPRS is stated on the label and shall be included in calculations used in the monograph and applicable general chapter(s). In cases where IPRS is not available with IPC, other equivalent pharmacopoeial standard may be used after establishing their suitability for intended purpose.

Impurity Reference Substances

Official applications of IP Impurity Reference Substances are specified in monographs and general chapters. Impurities Reference Substances may be presented as purified single-component materials or as mixture of more than one impurity.

Reference Standards for Vaccines

Considering the inherent variability in biological assays usage of reference standards is necessary at some stages in production of vaccines and antisera for their effective quality

control. Since these standards are key materials in establishing quality of products, they have to be properly established, maintained and monitored to ensure efficacy of the vaccines and antisera.

For many vaccines and antisera for human use, National Reference Standards (NRS) are established, maintained and supplied by the Central Drugs Laboratory (CDL), Kasauli. NRS are calibrated against the International Reference Standard (IRS) and are supplied to various vaccine and antisera manufacturers across the country. NRS are thus traceable to the IRS supplied by the National Institute for Biological Standards and Control, London. To ensure judicious use of NRS and IRS, manufacturers are encouraged to develop their *in-house* Reference Standards in comparison to the NRS and/ or IRS.

Microbial Reference Cultures

Microbial Reference Cultures are microbial preparations acquired from authentic Microbial Culture Collection Centre. They are very crucial and are required to carry out microbiological tests. These must be handled carefully because of their viability and characteristics that are dependent on proper handling and storage. The handling and storage of cultures by the laboratory should be done in a standardised way to avoid contamination and or alteration of growth characteristics. The number of transfers of cultures from original culture should be tracked to prevent excessive sub-culturing that increases the risk of phenotypic alteration or mutation.

Confirmation of the purity of the culture and identity of the culture should be performed at periodic intervals as per in-house procedure for the same. A list of microbial reference cultures used in various tests is described in Table 1 of chapter 2.2.20. Maintenance, Identification, Preservation and Disposal of Microorganisms.

Herbal Reference Substances

Herbal medicinal products are mixture complex, making them very difficult to identify. The situation get worsened by potential adulteration, whose identification using morphological characters is impracticable. Indian Pharmacopoeia Botanical Reference Substances abbreviated as IPBRS whose botanical identity has been established up to both genus and species level. The IPBRS mainly comprises of crude drugs, enabling users to confirm the authenticity of botanical samples through HPTLC/TLC fingerprint or using other specified test parameters described in the IP monograph. These IPBRS are appropriate for inteded uses in the standardization or quality control of herbs and herbal materials.

Performance Verification Reference Substances

These materials are provided by IPC to analyze and where appropriate to facilitate adjustment of the operation of an instrument to ensure that the results obtained are accurate and precise. The use of Performance Verification Reference Substances is generally described in associated general chapters and allied information.

Packaging

IPRS are dispensed into containers under appropriate filling and closure conditions to ensure the integrity of the reference substances. The quantity of IPRS supplied in each container is sufficient for use in tests or assays as specified in the monographs. Some substances (mainly materials with significant handling requirements or materials that are available only in small amounts) are provided in single-use containers. Such single-use containers generally are lyophilized, and their content is labeled in mass or activity units per container. If so labeled, the content of the container should be reconstituted in its entirety without any additional weighing.

Labelling

The label bears the name of the reference substance, the name and address of IPRS provider, catalogue number, Lot number and unit quantity (quantity per vial/ ampoule).The label includes an assigned value for substances with quantitative applications. Unless otherwise directed in the individual monograph or in a general chapter, IPRS should be used in accordance with the instructions on the label. IPRS do not carry an expiry date on the label. A lot of IPRS may be used in its official applications as long as it is listed as 'Current Lot' on IPC website.

Proper use

In the monographs, the tests and assays are based on comparison of a test solution with an IPRS and measurements are made on preparations of both the test and reference solutions. Where it is directed in a monograph that a reference solution of an IPRS be prepared for an assay or a test, it is intended that the standard shall be accurately weighed, taking into account the relatively large errors associated with weighing small quantities.

Storage

In order to serve the intended purpose, it is important that each IPRS is properly stored, handled and used. IPRS shall be stored in their original stoppered containers in a dry place, away from heat and protected from light. Storage conditions are given in certificate provided along with IPRS. Special storage conditions, where necessary, are usually given on the label. If an unopened container is stored in the recommended

conditions, it remains suitable for use as long as it is of the current lot. The user is responsible for ensuring that the contents of opened containers continue to be suitable for their intended use.

5.10. Elemental Impurities

This chapter specifies limits for the amounts of elemental impurities in drug products. Regardless of the approach used, compliance with the limits specified is required for all drug products unless otherwise specified in an individual monograph or specifically excluded in this chapter.

Elemental impurities include catalysts and environmental contaminants that may be present in drug substances, excipients, or drug products. These impurities may occur naturally, be added intentionally, or be introduced inadvertently (e.g., by interactions with processing equipment and the container–closure system). When elemental impurities are known to be present, have been added, or have the potential for introduction, assurance of compliance to the specified levels is required. A risk-based control strategy may be appropriate when analysts determine how to assure compliance with this standard. Due to the ubiquitous nature of arsenic, cadmium, lead, and mercury, they (at the minimum) must be considered in the risk assessment.

This chapter does not apply to radiopharmaceuticals; articles intended only for veterinary use; vaccines; cell metabolites; DNA products; allergenic extracts; cells, whole blood, cellular blood components or blood derivatives including plasma and plasma derivatives; products based on genes (gene therapy); cells (cell therapy); tissue (tissue engineering); dialysate solutions not intended for systemic circulation; total parenteral nutrition's (TPNs); elements that are intentionally included in the drug product for therapeutic benefit and nutraceuticals.

The limits presented in this chapter do not apply to excipients and drug substances, except where specified in an individual monograph. However, manufacturers of pharmaceutical products need certain information about the content of elemental impurities in drug substances or excipients in order to meet the criteria of this chapter. Drug product manufacturers can use elemental impurity test data on components from tests performed by drug substance or excipient manufacturers, who may provide test data, or if applicable, risk assessments. Elemental impurity data generated by a qualified supplier of drug product components are acceptable for use by a drug product manufacturer to demonstrate compliance with this chapter in the final drug product. Drug substance or excipient manufacturers who choose to perform a risk assessment must conduct that risk assessment using Table 2 in this chapter. Elements that are inherent in the nature of the material, as in the case of some naturally-sourced materials, must be considered in the risk assessment.

Speciation

The determination of the oxidation state, organic complex, or combination is termed "speciation". Each of the elemental impurities has the potential to be present in differing oxidation or complexation states. However, arsenic and mercury are of particular concern because of the differing toxicities of their inorganic and complexed organic forms.

The arsenic limits are based on the inorganic (most toxic) form. Arsenic can be measured using a total-arsenic procedure under the assumption that all arsenic contained in the material under test is in the inorganic form. Where the limit is exceeded using a total-arsenic procedure, it may be possible to show, via a procedure that quantifies the different forms, that the inorganic form meets the specification.

The mercury limits are based upon the inorganic (2^+) oxidation state. The methyl mercury form (most toxic) is rarely an issue for pharmaceuticals. Thus, the limit was established assuming the most common (mercuric) inorganic form. Limits for articles that have the potential to contain methyl mercury (e.g., materials derived from fish) are to be provided in the monograph.

Routes of exposure

The elements included in the tables below have been placed into three classes, based on their toxicity and likelihood of occurrence in the drug product. The classification scheme is intended to focus the risk assessment on those elements that are the most toxic but also have a reasonable probability of inclusion in the drug product, shown in Table 2.

The toxicity of an elemental impurity is related to its extent of exposure (bioavailability). The extent of exposure has been determined for each of the elemental impurities of interest for three routes of administration: oral, parenteral, and inhalational. These limits are based on chronic exposure. Consider the oral permissible daily exposures (PDEs) in Table 1 as a starting point in developing specific PDEs for other routes of administration, except where otherwise stated in the individual monograph.

Drug Products

The limits described in the third through fifth columns of Table 1 are the base daily dose PDEs of the elemental impurities of interest for a drug product taken by a patient according to indicated routes of administration.

Parenteral products. Parenteral drug products with maximum daily volumes up to 2 liter may use the maximum daily volume to calculate permissible concentrations from PDEs. For

products whose daily volumes, as specified by labeling and/or established by clinical practice, may exceed 2 litre (e.g., saline, dextrose, and solutions for irrigation), a 2- litre volume may be used to calculate permissible concentrations from PDEs.

Table 1 – Permitted Daily Exposures for Elemental Impurities

Element	Class	Oral PDE (µg/day)	Parenteral PDE (µg/day)	Inhalation PDE (µg/day)
Cadmium	1	5	2	3
Lead	1	5	5	5
Arsenic	1	15	15	2
Mercury	1	30	3	1
Cobalt	2A	50	5	3
Vanadium	2A	100	10	1
Nickel	2A	200	20	5
Thallium	2B	8	8	8
Gold	2B	100	100	1
Palladium	2B	100	10	1
Iridium	2B	100	10	1
Osmium	2B	100	10	1
Rhodium	2B	100	10	1
Ruthenium	2B	100	10	1
Selenium	2B	150	80	130
Silver	2B	150	10	7
Platinum	2B	100	10	1
Lithium	3	550	250	25
Antimony	3	1200	90	20
Barium	3	1400	700	300
Molybdenum	3	3000	1500	10
Copper	3	3000	300	30
Tin	3	6000	600	60
Chromium	3	11000	1100	3

Recommendations for Elements to be considered in the Risk Assessment

Table 2 identifies elemental impurities for inclusion in the risk assessment. This table can be applied to all sources of elemental impurities in the drug product.

Table 2 – Elements to be Considered in the Risk Assessment

Element	Class	If Intentionally Added (All Routes)	If Not Intentionally Added		
			Oral	Parenteral	Inhalation
Cadmium	1	Yes	Yes	Yes	Yes
Lead	1	Yes	Yes	Yes	Yes
Arsenic	1	Yes	Yes	Yes	Yes
Mercury	1	Yes	Yes	Yes	Yes
Cobalt	2A	Yes	Yes	Yes	Yes
Vanadium	2A	Yes	Yes	Yes	Yes
Nickel	2A	Yes	Yes	Yes	Yes
Thallium	2B	Yes	No	No	No
Gold	2B	Yes	No	No	No
Palladium	2B	Yes	No	No	No
Iridium	2B	Yes	No	No	No
Osmium	2B	Yes	No	No	No
Rhodium	2B	Yes	No	No	No
Ruthenium	2B	Yes	No	No	No
Selenium	2B	Yes	No	No	No
Silver	2B	Yes	No	No	No
Platinum	2B	Yes	No	No	No
Lithium	3	Yes	No	Yes	Yes
Antimony	3	Yes	No	Yes	Yes
Barium	3	Yes	No	No	Yes
Molybdenum	3	Yes	No	No	Yes
Copper	3	Yes	No	Yes	Yes
Tin	3	Yes	No	No	Yes
Chromium	3	Yes	No	No	Yes

Options for Demonstrating Compliance

A. Drug Product Analysis Option

The results obtained from the analysis of a typical dosage unit, scaled to a maximum daily dose, are compared to the Daily Dose PDE.

Daily Dose PDE ≥ measured value (µg per g) × maximum daily dose (g per day)

The measured amount of each impurity is not more than the Daily Dose PDE, unless otherwise stated in the individual monograph.

Based on this, calculation formula mentioned for limit is as follows.

$$\text{Measured value } \mu g \text{ per g (ppm)} \text{ (Specification Limit)} = \frac{\text{PDE } (\mu g \text{ per day})}{\text{Maximum daily dose (g per day)}}$$

Example for Limit

Elements	Class	PDE (µg per day)	Daily dose (g per day)	Specification Limit
Cadmium	1	5	10	0.5

B. Summation Option

Separately, add the amounts of each elemental impurity (µg per g) present in each of the components of the drug product:

$$\text{Daily Dose PDE} \geq \left[\sum_{1}^{M} (C_M \times W_M\right] \times D_D$$

Where, M = each ingredient used to manufacture a dosage unit;

C_M = element concentration in component (drug substance or excipient) (µg per g);

W_M = weight of component in a dosage unit (g per dosage unit);

D_D = number of units in the maximum daily dose (unit per day).

The result of the summation of each impurity is not more than the Daily Dose PDE, unless otherwise stated in the individual monograph. Before products can be evaluated using this option, the manufacturer must ensure that additional elemental impurities cannot be inadvertently added through the manufacturing process or via the container–closure system over the shelf life of the product.

C. Individual Component Option

For drug products with a daily dose of not more than 10 g, if all drug substances and excipients in a formulation meet the concentration limits shown in Table 3, then these components may be used in any proportion. No further calculation is necessary. While elemental impurities derived from the manufacturing process or the container–closure system are not specifically provided for in the Individual Component Option, it is expected that the drug product manufacturer will ensure that these sources do not contribute significantly to the total content of elemental impurities.

Drug Substance and Excipients

The acceptable levels of elemental impurities depend on the material's ultimate use. Therefore, manufacturers of pharmaceutical products need certain information about the content of elemental impurities in drug substances or excipients in order to meet the criteria of this chapter. Drug product manufacturers can use elemental impurity test data on components from tests performed by drug substance manufacturers or excipient manufacturers, who may provide test data, or, if applicable, risk assessments. Elemental impurity data generated by a qualified supplier of drug product components are acceptable for use by a drug product manufacturer to demonstrate compliance with this chapter in the final drug product. Drug substance or excipient manufacturers who choose to perform a risk assessment must conduct that risk assessment using Table 2 in this chapter. Elements that are inherent in the nature of the material, as in the case of some naturally-sourced materials, must be considered in the risk assessment.

The values provided in Table 3 are example concentration limits for components (drug substances and excipients) of drug products dosed at a maximum daily dose of 10 g per day. These values serve as default concentration limits to aid discussions between drug product manufacturers and the suppliers of the components of their drug products.

Table 3 – Permitted Concentrations of Elemental Impurities for Individual Component Option

Element	Class	Oral Concentration (µg/g)	Parenteral Concentration (µg/g)	Inhalation Concentration (µg/g)
Cadmium	1	0.5	0.2	0.3
Lead	1	0.5	0.5	0.5
Arsenic	1	1.5	1.5	0.2
Mercury	1	3	0.3	0.1
Cobalt	2A	5	0.5	0.3
Vanadium	2A	10	1	0.1
Nickel	2A	20	2	0.5
Thallium	2B	0.8	0.8	0.8
Gold	2B	10	10	0.1
Palladium	2B	10	1	0.1
Iridium	2B	10	1	0.1
Osmium	2B	10	1	0.1
Rhodium	2B	10	1	0.1
Ruthenium	2B	10	1	0.1
Selenium	2B	15	8	13
Silver	2B	15	1	0.7
Platinum	2B	10	1	0.1
Lithium	3	55	25	2.5
Antimony	3	120	9	2
Barium	3	140	70	30
Molybdenum	3	300	150	1
Copper	3	300	30	3
Tin	3	600	60	6
Chromium	3	1100	110	0.3

NOTE — Individual components may need to be limited at levels different from those in the table depending on monograph-specific mitigating factors.

If, by process monitoring and supply chain control, manufacturers can demonstrate compliance, then further testing may not be needed. When testing is done to demonstrate compliance, following procedures may be used.

Procedures

Determined by inductively coupled plasma- optical emission spectrometry (ICP-OES) or inductively coupled plasma- mass spectrometry (ICP-MS) (2.4.42).

Method A. Method can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–AES or ICP–OES).

Method B. Method can be used for elemental impurities generally amenable to detection by ICP–MS. Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the alternative procedure validation requirements below.

NOTE — System standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis.

Sample preparation

Forms of sample preparation include Neat, Direct aqueous solution, Direct organic solution, and Indirect solution. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any of the following appropriately validated preparation procedures. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with the same target elements, and where possible, using the same spiking solution. Reference solutions may contain multiple target elements.

NOTE — All liquid samples should be weighed.

Neat. Used for liquids or alternative procedures that allows the examination of unsolvated samples.

Direct aqueous solution. Used when the sample is soluble in an aqueous solvent.

Direct organic solution. Used where the sample is soluble in an organic solvent.

Indirect solution. Used when a material is not directly soluble in aqueous or organic solvents. Total metal extraction is the preferred sample preparation approach to obtain an Indirect solution. Digest the sample using the Closed vessel digestion procedure provided below or one similar to it. The sample preparation scheme should yield sufficient sample to allow quantification of each element at the limit specified in the corresponding monograph or chapter.

Closed vessel digestion. This sample preparation procedure is designed for samples that must be digested in concentrated acid using a closed vessel digestion apparatus. Closed vessel digestion minimizes the loss of volatile impurities. The choice of a concentrated acid depends on the sample matrix. The use of any of the concentrated acids may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times (*NOTE — Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used).*

For example. Dehydrate and predigest 0.5g of primary sample in 5 ml of freshly prepared concentrated acid. Keep for 30 minutes in a fume hood. Add an additional 10 ml of concentrated acid, and digest, using a closed vessel technique, until digestion or extraction is complete. Repeat, if necessary, by adding an additional 5 ml of concentrated acid.

NOTE — Where closed vessel digestion is necessary, follow the manufacturer's recommended procedures to ensure safe use.

Alternatively, leachate extraction may be appropriate with justification following scientifically validated metal disposition studies, which may include animal studies, speciation, or other means of studying disposition of the specific metal in the drug product.

Methods

NOTE — All reagents used for the preparation of test solution and reference solutions should be free of elemental impurities.

A. Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) (2.4.42).

Standardization solution 1. 1.5 J of the target element(s) in a matched matrix.

Standardization solution 2. 0.5 J of the target element(s) in a matched matrix.

Test solution (a). Proceed as directed in sample preparation above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

Test solution (b). Dilute test solution (a) with an appropriate solvent to obtain a final concentration of the target elements at not more than 1.5J.

Blank. Matched matrix

Elemental spectrometric system

- Mode. ICP,
- Detector. Optical detection system,
- Rinse. Diluent used.

Standardization. Standardization solution 1, standardization solution 2, and blank.

System suitability

Sample. Standardization solution 1

Suitability requirements

Drift. Compare results obtained from Standardization solution 1 before and after the analysis of the Sample solution.

Suitability criteria. Not more than 20 per cent for each Target element.

NOTE — If samples are high in mineral content, rinse system well before introducing the Sample in order to minimize carryover.

Analysis. Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size.

NOTE — Appropriate measures must be taken to correct for matrix induced interferences (e.g., wavelength overlaps).

B. Inductively Coupled Plasma- Mass Spectrometry (ICP-MS) (2.4.42).

Standardization solution 1. 1.5 *J* of the target element(s) in a matched matrix.

Standardization solution 2. 0.5 *J* of the target element(s) in a matched matrix.

Test solution (a). Proceed as directed for sample preparation above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

Test solution (b). Dilute test solution (a) with an appropriate solvent to obtain a final concentration of the target elements at not more than 1.5 J.

Blank. Matched matrix.

Elemental spectrometric system

- Mode. ICP, [*NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)*]
- Detector. Mass spectrometer,
- Rinse. Diluent used.

Standardization. Standardization solution 1, standardization solution 2 and blank.

System suitability

Sample. Standardization solution 1.

Suitability requirements

Drift. Compare results obtained from standardization solution 1 before and after the analysis of the sample solution.

Suitability criteria. Drift not more than 20 per cent for each Target element.

NOTE—If samples are high in mineral content, rinse system well before introducing the Sample in order to minimize carryover.

Analysis. Analyze according to the manufacturer's suggestions for program and *m/z*. Calculate and report results based on the original sample size.

NOTE — Appropriate measures must be taken to correct for matrix-induced interferences (e.g. argon chloride interference with arsenic determinations).

Use of Alternative Procedures

Alternative procedures that meet the validation requirements herein may be used in accordance with General Notices. Information on the requirements for alternate procedure validation is as follows.

Requirements for Alternate Procedure Validation

NOTE — Some validation requirements provided below may differ from those provided in chapter 2.4.2 and 2.4.42.

If the specified pharmacopoeial procedures do not meet the needs of a specific application, an alternative procedure may be developed. Alternative procedures must be validated and shown to be acceptable, in accordance with the validation requirements for alternative procedures as described below. The level of validation necessary to ensure that an alternative procedure is acceptable depends on whether a limit test or a quantitative determination is specified in the monograph. The requirements for the validation of an elemental impurities procedure for each type of determination are described below. Any alternative procedure that has been validated and meets the acceptance criteria that follow is considered to be suitable for use.

Limit Procedures

The following section defines the validation parameters for the acceptability of alternative limit procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference material.

The suitability of the method must be determined by conducting studies with the material or mixture under test supplemented with known concentrations of each target element of interest at the appropriate acceptance limit concentration. The material or mixture under test must be spiked before any sample preparation steps are performed.

Detectability

Reference solution. A preparation of reference materials for the target element(s) at the target concentration.

Spiked test solution (a). Prepare a solution of sample under test, spiked with appropriate reference materials for the target elements at the target concentration, solubilized or digested as described in sample preparation.

Spiked test solution (b). Prepare a solution of the sample under test, spiked with appropriate reference materials at 80 per cent of the target concentration for the target elements, solubilized or digested as described in sample preparation.

Unspiked test solution. A sample of material under examination, solubilized or digested in the same manner as the sample solutions.

For non-instrumental procedures, spiked test solution (a) provides a signal or intensity equivalent to or more than that of the reference solution. Spiked test solution (b) must provide a signal or intensity less than that of spiked test solution (a) (*NOTE — The signal from each spiked test solution is not less than the unspiked test solution determination).*

For instrumental procedures, the average value of the three replicate measurements of spiked test solution (a) is within ± 15 per cent of the average value obtained for the replicate measurements of the reference solution. The average value of the replicate measurements of spiked test solution (b) must provide a signal intensity or value less than that of the reference solution (*NOTE — Correct the values obtained for each of the spiked solutions using the unspiked test solution*).

Precision for Instrumental Methods (Repeatability)

NOTE — Non-instrumental precision is demonstrated by meeting the detectability requirement above.

Test solutions. Six independent samples of the material under test, spiked with appropriate reference materials for the target elements at the target concentration.

The relative standard deviation is not more than 20 per cent for each target element.

Specificity

The procedure must be able to unequivocally assess (2.5.10 Validation of Analytical Procedures) each target element in the presence of components that may be expected to be present, including other target elements, and matrix components.

Quantitative Procedures

The following section defines the validation parameters for the acceptability of alternative quantitative procedures. Meeting these requirements must be demonstrated experimentally, using an appropriate system suitability procedure and reference materials. Meeting these requirements demonstrates that the procedure is equivalent to the pharmacopoeial procedure for the purpose of quantifying the target elements.

Accuracy

Test solutions. Prepare samples of the material under test spiked with appropriate reference materials before any sample preparation steps (digestion or solubilization) at concentrations ranging from 50 per cent to 150 per cent of J for each target element.

Reference solutions. Prepare solutions containing the target elements at concentrations ranging from 50 per cent to 150 per cent of J, using appropriate reference materials.

The spike recovery is between 70 per cent to 150 per cent for the mean of three replicate preparations at each concentration.

Precision

Repeatability

Test solutions. Six independent samples of material under examination (taken from the same lot) spiked with appropriate reference materials for the target element(s) at the indicated level.

The relative standard deviation is not more than 20 per cent (N = 6) for each target element.

Intermediate precision (ruggedness)

Perform the repeatability analysis again either on a different day, with a different instrument, with a different analyst, or a combination thereof. Combine the results of this analysis with the repeatability analysis so the total number of analyses is 12.

The relative standard deviation is not more than 25 per cent (N = 12) for each target element.

Specificity

The procedure must be able to unequivocally assess (2.5.10 Validation of Analytical Procedures) each target element in the presence of components that may be expected to be present, including other target elements, and matrix components.

Limit of Quantitation, Range and Linearity

Complied by meeting the accuracy requirement.

Definition of Terms	
Concentrated acid	Concentrated ultra-pure nitric, sulphuric, hydrochloric, or hydrofluoric acids or Aqua regia (a mixture of concentrated hydrochloric and nitric acids, at ratios of 3:1 or 4:1, respectively).
Matched matrix	Solutions having the same solvent composition as the test solution. In the case of an aqueous solution, matched matrix would indicate that the same acids, acid concentrations, and mercury stabilizer are used in both preparations.

Contd.

Definition of Terms	
Target elements	Elements with the potential of being present in the material under test. Include arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) in the target element evaluation when testing is done to demonstrate compliance. Target elements should also include any elements that may be added through material processing or storage.
Target limit or Target concentration	The acceptance value for the elemental impurity being evaluated. Exceeding the target limit indicates that a material under test exceeds the acceptable value. Target limits can be approximated by dividing the *Daily Dose PDEs* by the maximum daily dose for the *Drug Product Analysis Option*.
J	The concentration (w/w) of the element(s) of interest at the Target limit, appropriately diluted to the working range of the instrument. For example, if the target elements are lead and arsenic for an analysis of an oral solid drug product with a daily dose of 10 g per day using inductively coupled plasma–mass spectrometry (ICP–MS), the target limit for these elements would be 0.5 µg per g and 1.5 µg per g (Table 2). However, in this case, the linear dynamic range of the ICP–MS is known to extend from 0.01 ng per ml to 0.1 µg per ml for these elements. Therefore, a dilution factor of at least 1:100 is required to ensure that the analysis occurs in the linear dynamic range of the instrument. *J* would thus equal 5 ng and 15 ng per ml for lead and arsenic, respectively, when the dilution factor is added.

Based on above, calculation formula for J value is simplified as follows.

$$\text{J Value} = \frac{\text{PDE } (\mu\text{g per day})}{\text{Maximum daily dose (g per day)}} \times \text{Sample concentration in g per ml}$$

Below example given for calculation of target value in ppm with respect to sample concentration 0.002g per ml

Elements	Class	PDE (µg per day)	Daily dose (g per day)	Sample concentration (g per ml)	J value in ppm	J value in ppb
Cadmium	1	5	10	0.002	0.001	1

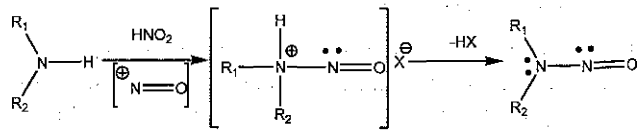
5.11. Nitrosamine Impurities

Nitrosamines became popular when there was recall of angiotensin II receptor blocker (ARB) medicines, known as "sartans", due to the presence of an impurity, N-nitrosodimethylamine (NDMA). Valsartan and Losartan are the worst affected and several lots of these products have been recalled globally. N-nitroso compounds are among the structural groups having high potency mutagenic carcinogens in several animal species, and some are classified as probable or possible human carcinogens referred to as the "cohort of concern" in ICH M7(R1): Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. So, these impurities must be controlled at or below the acceptable cancer risk. This family of impurities has potential for Genotoxicity, hence pose an additional safety concern to patient and as a result of the potential toxicity associated, it is recommended to take steps to control and limit their presence in pharmaceutical materials.

This chapter has been introduced in IP to provide guidance for the control of nitrosamine impurities in order to ensure that the potential presence of nitrosamines in drug substances and drug products is identified, assessed and controlled.

Formation of Nitrosamines

Nitrosamines are a group of compounds having the chemical structure of nitroso group bonded to an amine (R1N(-R2)-N=O). They are formed by reaction between secondary, tertiary, or quaternary amines and nitrous acid (nitrite salts under acidic conditions) by nitrosating reaction.

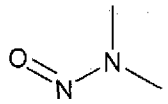
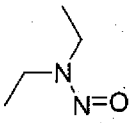
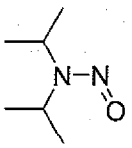
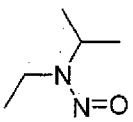
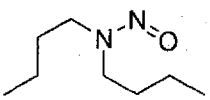
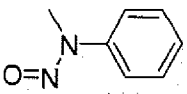
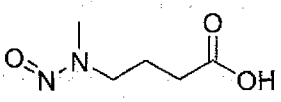


Nitrosamine Formation Reaction

Source of Nitrosamines

There are a number of pathways by which nitrosamines can be introduced into or generated as impurities in pharmaceutical drug products. Specifically, nitrosamines are formed by chemical reaction of secondary, tertiary or quaternary amines with nitrites under acidic conditions. Under these conditions,

Table 1 – Nitrosamines Found as Contaminants in Drug Substances and Drug Products

Common Name and Chemical Name	Acronym	CAS #	Structure	Chemical Formula	Molecular Weight
Nitrosodimethylamine *N*-Methyl-*N*-nitrosomethanamine	NDMA	62-75-9		$C_2H_6N_2O$	74.08
Nitrosodiethylamine *N*-Ethyl-*N*-nitrosoethanamine	NDEA	55-18-5		$C_4H_{10}N_2O$	102.14
Nitrosodiisopropylamine *N*-Isopropyl-*N*-nitrosoisopropylamine	NDIPA	601-77-4		$C_6H_{14}N_2O$	130.19
Nitrosoethylisopropylamine *N*-Ethyl-*N*-nitroso-2-propanamine	NEIPA	16339-04-1		$C_5H_{12}N_2O$	116.16
Nitrosodibutylamine *N*-Butyl-*N*-nitroso-1-butanamine	NDBA	924-16-3		$C_8H_{18}N_2O$	158.25
Nitrosomethylphenylamine *N*-Methyl-*N*-nitrosophenylamine	NMPA	614-00-6		$C_7H_8N_2O$	136.15
Nitrosomethylaminobutyric acid 4-[Methyl(nitroso)amino] butanoic acid	NMBA	61445-55-4		$C_5H_{10}N_2O_3$	146.15

nitrite salts may form nitrous acid, which can react with an amine to form a nitrosamine (See Formation of Nitrosamines). Some examples of the reported sources or pathways leading to the generation of nitrosamines identified empirically or reported include (but are not limited to) the following:

Nitrosating Agents

+NO	Nitrosonium Ion
Phenol-NO	Nitrosyl Phenol
Halide-NO	Nitrosyl Halide
HNO_2	Nitrous Acid
ONO-NO (N_2O_3)	Nitrous Anhydride
CNS-NO	Nitrosyl Thiocyanate
O_2.NO-NO (N_2O_4)	Dinitrogen Tetraoxide
Y.NO(SO_4-ClO_4^-)	Nitrosyl Sulfate/Perchlorate

Sources of Secondary, Tertiary, Quaternary Amines and nitrite salts that can form nitrosamines

- The API (or API degradants), intermediates, or starting materials may contain secondary or tertiary amine functional groups. Tertiary and quaternary amines used as reagents in the synthesis of APIs may contain other amine impurities. Tertiary amines, such as triethylamine, have been shown to contain low levels of other secondary amines (such as dipropylamine and isopropyl ethylamine). Secondary and tertiary amines may be present as impurities or degradants formed by dealkylation of quaternary amines.
- The drug substance itself, which may degrade under some conditions resulting in the formation of nitrosamines (e.g., ranitidine).

— Impurities in the container–closure system for the finished drug product, which may include impurities capable of forming nitrosamines, especially if associated with materials containing amines and potential sources of a nitrosating agent (e.g., nitrite, nitrocellulose).

Contamination in Vendor-Sourced Raw Materials

— In contaminated vendor-sourced materials, like starting materials and raw materials, nitrosamine impurities can be formed. Nitrosamine contamination takes place when fresh solvents (*o*-xylene, toluene, and methylene chloride) were contaminated during shipment from vendors (e.g., during transfer between storage vessels).

— Cross-contamination of starting materials may takes place if they are manufactured at sites where nitrosamine impurities are produced in other processes.

— Sodium nitrite present in starting materials reacts with amines under acidic conditions to form nitrosamines. Special attention should be given to the formation of nitrogen-containing heterocycles by employing azide followed by quenching with nitrous acid to remove excess azide.

— Impurities like secondary or tertiary amines have been reported in some raw materials and in fresh solvents like toluene.

Recovered Solvents, Catalysts, and Reagents as Sources of Contamination

— Due to the presence of residual amines (such as trimethylamine or diisopropylethylamine), recovered solvents, catalysts, and reagents may pose a risk of nitrosamine impurities.

— Raw materials can be contaminated, if adequate cleaning of equipment between customers, or between different materials, is not carried out or is not validated as capable of removing each impurity of concern.

Lack of Process Optimization and Control

— Potential source for the formation of nitrosamine impurities are lack of optimization of the manufacturing process for APIs when reaction conditions such as temperature, pH, or the sequence of adding reagents, intermediates, or solvents are inappropriate or poorly controlled.

— A risk assessment should be conducted to determine the materials that contribute to the potential for inclusion of nitrosamines in the drug products. All potential sources for the introduction of nitrosamines should be considered in the risk assessment including the drug substance, excipients, water, solvents, the manufacturing process, packaging components, and formation on stability.

Table-2. Some of the identified risks associated with several of the potential sources of nitrosamines are as follows:

Potential sources of Nitrosamines	Observed Risk
Solvents	— Presence of residual dialkylamines or tri-substituted amines that can degrade to form intermediates that can further react with nitrosating agents. — Presence of nitrites or other nitrosating agents. — Presence of acid. — Limited controls/specification limits for recycled solvents. — Poor quality solvents.
Water	— Presence of residual dialkylamines or impurities that can degrade to form dialkyl amines. — Presence of acid and nitrosating agents.
Excipients	— Presence of nitrites or other nitrosating agents and/or nitrosamine impurities.
Drug substance	— Use of sodium azide in the synthesis followed by use of nitrites in acidic medium (nitrous acid) for quenching excess azides — Use of di- or tri-alkylamines and amides (e.g., dimethyl-formamide [DMF], dimethylamine [DMA], triethylamine[TEA], *N*-methyl-pyrrolidone [NMP]) in the presence of nitrites and acid media. — Use of recycled solvents that may contain nitrosamine or their precursors. — Use of sanitized water (e.g., chloramines). — Insufficient purification. — Degradation of drug substances containing functional groups that can then participate in nitrosation reactions.
Manufacturing process	— Contamination. — Use of recycled solvents that may contain nitrosamine or their precursors.

	— Presence of nitrous oxides in air used to dry the drug substance or drug product. — Carryover of relevant reactive species into subsequent steps.
Drug Product (Including stability)	— Secondary, tertiary, or quaternary amine group in molecule of drug substance. — Presence of nitrate counter ions (potentially containing nitrite as an impurity). — Potential reactions within the formulation matrix during stability/ shelf life (e.g., presence or generation of acidic conditions, moisture and heat).
Container-closures	— Packaging materials containing vulnerable amines that might react with nitrosating agents present in the packaging material itself (e.g., amines in inks reacting with nitrocellulose print base).

Nitrosamine Risk Assessments-Development of a Control Strategy

In order to maintain the nitrosamine impurities at or below the level of acceptable intake, certain level of control is needed by the drug product manufacturer to determine the potential formation or contamination with nitrosamine impurities (Manufacturers may refer to the ICH guidance for industry Q9 Quality Risk Management).

Although following different sources with the highest potential for nitrosamines should also be included in risk assessment to establish if controls or additional controls are needed.

— Drug substance synthetic route,

— Drug substance manufacturing process,

— Drug substance manufacturing route,

— Excipients,

— Raw materials.

In all cases, if nitrosamines are predicted by the risk assessment or confirmed to be present through testing of the drug substance, drug product, or other materials, a control strategy should define an approach to ensure that the nitrosamine levels comply with the established acceptable intakes (AIs) and should be aligned with the current regulatory requirements in place (Fig. 5.11-1).

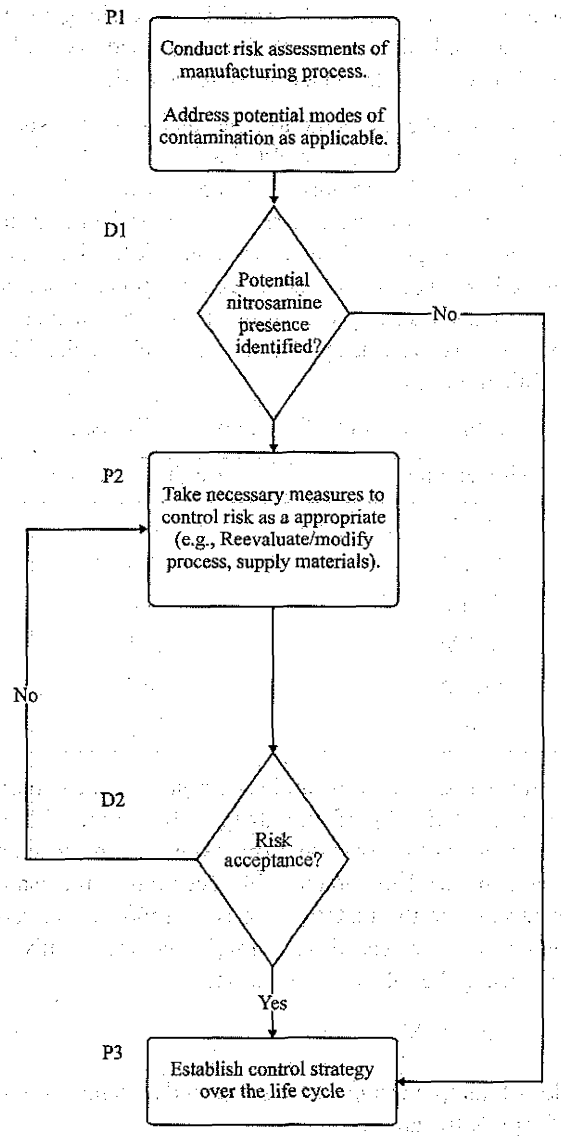


Fig. 5.11-1: High level process for development of a nitrosamine impurity controls strategy.
P1, P2, P3 = Process 1, 2, 3; D1, D2 = Decision 1, 2

Limits of Nitrosamines

The Acceptable intake (AI) limit defined as an intake level that poses a negligible health risk, is a daily exposure to a compound such as NDMA, NDEA, NMBA, NMPA, NIPEA, or NDIPA that approximates a 1:100,000 cancer risk after 70 years of exposure. The term acceptable intake (AI) is used in ICH M7(R1) to indicate the threshold of toxicological concern (TTC) considered for the impurity to be associated with negligible risk of carcinogenicity or other toxic effects. However, it does not apply for nitrosamines because they are among the structural groups of high potency mutagenic carcinogens and intake below the TTC would theoretically be

associated with a potential for significant carcinogenic risk. Hence, the available safety data should be used to establish a material specific intake on a case-by-case basis.

There are a number of methodologies that toxicologists have applied in establishing acceptable intakes (AIs). In this case, the median tumorigenic dose (TD_{50}) of NDMA, NDEA, and other nitrosamines is used as representative data in a linear extrapolation to establish an acceptable risk level. AI can be calculated based on rodent carcinogenicity potency data such as TD_{50} values (doses giving a 50 per cent tumor incidence equivalent to a cancer risk probability level of 1:2) identified in the public literature.

Table 3- AI Limits for Guidance of Industry

Nitrosamine	AI Limit (ng per day)
NDMA	96
NDEA	26.5
NMBA	96
NMPA	26.5
NIPEA	26.5
NDIPA	26.5

Since the exposure to nitrosamines is related to the maximum daily dose (MDD) of the drug substance, different concentrations of nitrosamines (ng per g) may be acceptable for each material evaluated. The acceptable nitrosamine concentration in the material varies by product and can be calculated in ppm, based on a drug's maximum daily dose (MDD) using the following equation:

AI (ng)/MDD (mg)

Table 4- Example Using AI Limits 96 and 26.5 ng per day for the Target Nitrosamines

Active substance (Maximum daily dose)	NDMA		NDEA	
	AI (ng/ day	Limit (ppm)	AI (ng/ day	Limit (ppm)
Candesartan (32 mg)	96	3.00	26.5	0.83
Irbesartan (300 mg)		0.32		0.088
Losartan (100 mg)		0.96		0.27
Olmesartan (40 mg)		2.40		0.66
Valsartan (320 mg)		0.30		0.083

These limits are applicable only if a drug substance and drug product contains a single nitrosamine. If more than one of the nitrosamine impurities identified in Table 1 is detected and the total quantity of nitrosamine impurities exceeds 26.5 ng per day (the AI for the most potent nitrosamines) based on the maximum daily dose (MDD), the manufacturer should contact and consult with the relevant health authority or the regulatory authorities for evaluation to determine the specific path forward.

For drug products with an MDD of less than 880 mg per day, a recommended limit for total nitrosamines of 0.03 ppm is not more than 26.5 ng per day and is considered acceptable. For drug products with an MDD above 880 mg per day, the limit for total nitrosamines should be adjusted so as not to exceed the recommended limit of 26.5 ng per day.

If nitrosamines AI limits are not found for a drug product, manufacturers should use the approach outlined in ICH M7 (R1) to determine the risk associated with the nitrosamine and contact the regulatory authorities about the acceptability of any proposed limit.

Testing for the Presence of Nitrosamines

Upon completion of the risk assessment, exploratory testing may need to be performed to confirm the conclusions of the risk assessment and proposed control strategy. If a risk of nitrosamines in a drug product is identified, confirmatory testing of batches should be conducted using sensitive and appropriately validated methods. If a nitrosamine impurity is detected, manufacturers should investigate the root cause and implement changes in the manufacturing process to mitigate or reduce nitrosamine impurities.

Test Method Performance Characteristics

The AIs associated with nitrosamines require the application of sensitive analytical procedures. In many cases, the most reliable procedures take advantage of the sensitivity and selectivity of chromatographic separation techniques coupled with quantitation by mass spectrometry (e.g., HPLC–MS/MS, GC–MS/MS). Appropriate sample preparation is a critical step in trace impurity analyses such as those required to evaluate the levels of nitrosamines in drug substances or drug products and this is also particularly critical to prevent the loss or generation of nitrosamines.

The recommended method performance characteristics that need to be evaluated for quantitative analysis of nitrosamines include range, accuracy, repeatability, intermediate precision, and limit of quantitation. If a limit test is intended for use, the recommended method performance characteristics to be assessed include specificity, recovery, detectability, and solution stability. The performance criteria for these parameters should be properly set and confirmed through validation to ensure that the method is suitable for its intended use based on the specific analytes, matrices, and required precision and accuracy of the analytical procedures. Precision and recovery depend highly on concentration and matrix complexity, and the final proposed acceptance criteria need to be justified in the procedure of validation documentation.

Generally, sensitive methods with limits of quantitation (LOQ) in the parts-per-billion (ppb) range are needed to meet the low AIs recommended for nitrosamines. Manufacturers of APIs and drug products should use methods with LOQs at or below 0.03 ppm. Manufacturers should establish methods for which the LOQ and limit of detection (LOD) are as low as reasonably practical for products for which the maximum daily dose is high (e.g., more than 1 g). If more than one nitrosamine listed in Table 1 is detected, then the analytical method should be validated for LOQs below 0.03 ppm to accurately quantify a total nitrosamine level of not more than 26.5 ng per day. For example, if the MDD is 1200 mg, the LOQ should be below 0.02 ppm.

Analytical Procedures

The following procedures have been provided as suitable for their intended (specified) purpose. Users should validate these methods while considering the effect of sample solubility and extraction efficiency on the test results for other materials for which they are intended to be applied.

Method A. Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, NMPA, and NDBA in selected sartans (valsartan, irbesartan, and losartan potassium) by HPLC–HRMS

Test solution: Dissolve 100 mg of substance under examination in *methanol* with the aid of vortex and dilute to 5.0 ml with *methanol*. Filter through a suitable filter of 0.2µm.

Reference solution (a). A solution containing 1.0 ng per ml, each of, *N-Nitrosodimethylamine IPRS, N-Nitrosodiethylamine IPRS, N-Nitrosoethylisopropylamine IPRS, N-Nitrosodiisopropylamine IPRS, N-Nitrosodibutylamine IPRS, N-Nitrosomethylphenylamine IPRS and N-Nitrosomethylaminobutyric Acid IPRS* in *methanol.*

Reference solution (b). A solution containing 6.0 ng per ml, each of, *N-Nitrosodimethylamine IPRS, N-Nitrosodiethylamine IPRS, N-Nitrosoethylisopropylamine IPRS, N-Nitrosodiisopropylamine IPRS, N-Nitrosodibutylamine IPRS, N-Nitrosomethylphenylamine IPRS and N-Nitrosomethylaminobutyric Acid IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with pentafluorophenyl groups bonded to porous silica (2.6 µm) (Such as Kinetex F5 100Å, phenomenex),
- column temperature: 40°,
- sampler temperature: 3°,
- mobile phase: A. 0.1 per cent v/v *formic acid* in *water*, B. 0.1 per cent v/v *formic acid* in *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 0.6 ml per minute,
- flow rate to ion source: 0.6 ml per minute,
- detector: high resolution mass spectrometer,
- injection volume: 3 µl.

MS conditions

- ionization: Electrospray ionization (ESI)
- scan settings: See below table-5

Table 5

Nitrosamine impurity	NDMA	NMBA	NDEA	NEIPA	NDIPA	NMPA	NDBA
Scan type	SIM[1]	SIM	PRM[2]	SIM	SIM	SIM	PRM
Polarity	positive	negative	positive	positive	positive	positive	positive
Scanstart–end (min)	1.0-3.5	3.5-5.5	5.5-7.0	7.0-8.5	8.5-10.0	8.5-10.0	13.0-15.5
m/z[3] isolated for PRM	-	-	103.0866	-	-	-	159.1492
NCE[4]	-	-	25	-	-	-	20
Scan range (m/z)	74.3-75.8	144.3-145.8	50.0-114.0	116.4-117.9	130.4-131.9	136.3-137.8	50.0-170.0
Microscans	3	3	3	3	3(1)[5]	3(1)[5]	3
Resolution	30,000	60,000	30,000	60,000	60,000	60,000	30,000
AGC target value (per cent)[6]	250	250	250	250	250	250	250

[1]SIM-selected ion monitoring,

[2]PRM - parallel reaction monitoring,

[3]m/z - mass to charge ratio,

[4]NCE -normalized collision energy,

[5]1-microscan must be used only if both NDIPA and NMPA are present,

[6]AGC - automatic gain control.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
1.5	90	10
7	45	55
17	45	55
17.1	10	90
21	10	90
21.1	90	10
25	90	10

NOTE — Divert the drug substance from the MS source during the elution.

Impurity name	Relative retention time
NDMA	0.20
NMBA	0.31
NDEA	0.46
NEIPA	0.57
NDIPA	0.66
NMPA	0.67
NDBA	1.00

Data processing. Peak areas in the extracted ion chromatograms (EIC) with an m/z extraction window of ±15 ppm are used for quantitation. The m/z values extracted are listed in Table 6.

NOTE — NMBA and NEIPA exist as syn-and anti-conformers due to the restricted rotation of the N–N bond. These conformers are partially separated by the method's chromatographic conditions. The NMBA peak is observed as a doublet. Integrate both of the NMBA peaks and use the combined peak areas for calculation of the NMBA concentration. The NEIPA peak may appear as a doublet or a single peak with a tailing shoulder. For NEIPA, if the conformers are resolved, integrate both peaks and combine the peak areas for the calculation of the NEIPA concentration. If the NEIPA conformers are not fully resolved (e.g., evidence of a shoulder is present), integrate the main peak and shoulder as a single peak and use the combined peak area to calculate the NEIPA concentration.

Inject reference solution (a) and reference solution (b). The test is not valid unless the signal-to-noise ratio for the principal peaks is not less than 10 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more 20.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the nitrosamine impurity concentration in the drug substance in ppm (µg per g).

Method B. Quantitation of NDMA, NDEA, NDIPA and NEIPA in selected sartans (valsartan, irbesartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan) by Headspace GC-MS.

Blank solution. Dissolve 100 mg of *imidazole* into a headspace vial, and then add 1.0 ml of internal standard solution and 1.0 ml of *acetonitrile*. Apply the stopper, cap, and crimp tightly.

Internal standard stock solution. A 0.4 µg per ml solution of *NDMA-d6 IPRS* in *methanol*.

Internal standard solution. Dilute 2.0 ml of the internal standard stock solution to 50.0 ml with *methanol*.

Test solution. Dissolve 200 mg of substance under examination and 100 mg of imidazole into a headspace vial, and then add 1.0 ml of internal standard solution and 1.0 ml of *acetonitrile*. Apply the stopper, cap, and crimp tightly.

Reference solution (a). A solution containing 0.4 µg per ml of, each *of, N-Nitrosodimethylamine IPRS, N-Nitrosodiethylamine IPRS, N-Nitrosoethylisopropylamine IPRS, and N-Nitrosodiisopropylamine IPRS* in *methanol*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) and 2.0 ml of internal standard stock solution to 50 ml in *methanol*.

Reference solution (c). Transfer 1.0 ml of reference solution (b) to a headspace vial containing 100 mg of imidazole and 1.0 ml of *acetonitrile*. Apply the stopper, cap, and crimp tightly.

Reference solution (d). A solution containing 0.004 µg per ml of, each *of, N-Nitrosodimethylamine IPRS, N-Nitrosodiethylamine IPRS, N-Nitrosoethylisopropylamine IPRS, and N-Nitrosodiisopropylamine IPRS in methanol* by diluting 0.5 ml of the reference solution (a) and 2.0 ml of internal standard stock solution to 50 ml with *methanol*.

Table 6

Nitrosamine Impurity	NDMA	NMBA	NDEA	NEIPA	NDIPA	NMPA	NDBA
m/z to be extracted	75.0553	145.0619	75.0553, 103.0866	117.1022	131.1179	137.0709	57.0704, 103.0872, 159.1492

Reference solution (e). Transfer 1.0 ml of reference solution (d) to a headspace vial containing 100 mg of imidazole and 1.0 ml of *acetonitrile*. Apply the stopper, cap, and crimp tightly.

Chromatographic system

- Mode: GC
- Injector : Headspace (See below table for Parameters)

Equilibration temperature	95°–110°
Loop temperature	150°
Rate 1	10° per minute
Transfer line temperature	160°
Pressurizing gas pressure	20.00 psi
Equilibration time	10.00 minutes
Pressurizing time	2.00 minutes
Load time	2.00 minutes
Injection time	1.00 minutes
Vial size	20 ml

- a fused silica column 30 m x 0.32 mm, coated with polyethylene glycol compound (avg. mol. weight about 15,000) (film thickness 1.0 µm) (Such as Supelcowax 10 or SH-Stabilwax),
- split ratio: 1:1 or 1:3 (Note- Split ratio can be modified to optimize sensitivity),
- mass spectrometer detector,
- temperature: See below table

Initial Temperature (°)	Temperature Ramp (° per min.)	Final Temperature (°)	Hold Time at Final Temperature (min.)
45	0	45	3
45	10	130	3
130	15	190	—
190	40	240	10

- flow rate: 1.8 ml per minute, using helium as carrier gas (adjustment and verification are necessary for other carrier gases),
- purge: 3.0 ml per minutes or default value,

MS conditions

Ionization mode	Electron impact (EI)
Polarity	positive
Event 1	
Name	NDMA
Start time	10.0 min
End time	12.0 min
Acquisition mode	multiple reaction mode (MRM)
Ch 1 m/z	74.00 > 44.00
CH 1 collision energy	4.00 V
Ch 2 m/z	74.00 > 42.00
CH 2 collision energy	15.00 V
Event 2	
Name	NDMA-d6
Start time	10.0 min
End time	12.0 min
Acquisition mode	MRM
Ch 1 m/z	80.00 > 50.00
CH 1 collision energy	5.00 V
Event 3	
Name	NDEA
Start time	12.00 min
End time	12.75 min
Acquisition mode	MRM
Ch 1 m/z	102.00 > 85.1
CH 1 collision energy	6.00 V
Ch 2 m/z	102.00 > 56.1
CH 2 collision energy	15.00 V
Event 4	
Name	NEIPA
Start time	12.75 min
End time	13.35 min
Acquisition mode	MRM
Ch 1 m/z	116.00 > 99.10
CH 1 collision energy	6.00 V
Ch 2 m/z	99.00 > 44.10
CH 2 collision energy	9.00 V
Event 5	
Name	NDIPA
Start time	13.35 min
End time	14.00 min
Acquisition mode	MRM
Ch 1 m/z	130.00 > 42.00
CH 1 collision energy	10.00 V
Ch 2 m/z	130.00 > 43.10
CH 2 collision energy	18.00 V

NOTE— Ch 1 m/z in multiple reaction modes (MRM) is used for quantitation

Inject the blank solution, reference solution (e) and reference solution (c).

Impurity name	Relative retention time
NDMA	0.80
NDMA-d6	0.80
NDEA	0.90
NEIPA	0.96
NDIPA	1.00

There should be no interfering peak in the Blank. The test is not valid unless, the signal-to-noise ratio for impurity peaks in the chromatogram obtained with reference solution (e) is not less than 10 and the ratio of the impurity standard peak response to the internal standard peak response from six replicate injections in the chromatogram obtained with reference solution (e), is not more than 20.0 per cent.

Inject reference solution (c) and the test solution.

Calculate the nitrosamine impurity concentration in the drug substance in ppm (µg per g).

Method C. Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA and NDBA in selected sartans (valsartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan) by HPLC-MS/MS.

solvent mixture. 1 per cent v/v *formic acid* in *water.*

Internal standard solution. A solution containing 10 µg per ml of, each *of, NDMA-d6 IPRS* and *NMBA-d3 IPRS*, and 1µg per ml of, each *of, NDEA-d10 IPRS* and *NDBA-d18 IPRS* in *water.*

Test solution. Dissolve 80 mg of substance under examination in lidded centrifuge tube. Add 1188 µl of the solvent mixture and 12 µl of internal standard solution. Vortex at 2500 rpm for 20 minutes (except for losartan potassium, which should be vortexed not more than 5 minutes). Centrifuge at about 10,000 rpm for 10 minutes and filter using PTFE filter 0.45µm.

Reference solution (a). A solution containing 200 ng per ml, each of, *N-nitrosodimethylamine IPRS, N-nitrosoethylisopropylamine IPRS, N-nitrosodiisopropylamine IPRS, N-nitrosodibutylamine IPRS,* and *N-nitrosomethylaminobutyric acid IPRS* in *water.*

NOTE — Store reference solution (a) mixture in amber vials at –18° to –20°.

Reference solution (b). A 132 ng per ml solution of *N-nitrosodiethylamine IPRS* in *water.*

Reference solution (c). Depending on the targeted nitrosamine concentration in the test solution, prepare a set of 5 consecutive linearity solutions from below table 7 from the reference solution (a) and reference solution (b) by mixing specified volumes of each solution as indicated.

NOTE — Following table 7 represents an example for preparing solutions for constructing the calibration curve. Other dilution schemes may be used for preparing the set of 5 linearity solutions covering the range of interest. L1 is used only for NDEA when applicable. For others, linearity starts with L2.

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (2.7 µm) (Such as ARC-18, Restek Raptor),
- mobile phase: A. 0.1 per cent v/v *formic acid* in *water,*
  B. 0.1 per cent v/v *formic acid* in *methanol,*
- flow rate: 0.5 ml per minute,

Table 7

Linearity solution #	Concentration level	Concentration of NDMA, NMBA, NDBA, NEIPA, NDIPA (ng/ml)/ NDEA (ng/ml)	Content of NDMA, NMBA, NDBA, NEIPA, NDIPA(ppb)/ NDEA (ppb)	Volume of Nitrosamine standard stock solution mixture (µl)	Volume of NDEA standard stock solution (µl)	Volume of water (µl)	Volume of internal standard (µl)	Total volume (µl)
1	L1	1.33/0.66	19.95/10	8	6	1174	12	1200
2	L2	2/0.88	30/13.5	12	8	1168	12	1200
3	L3	5/3.3	75/49.5	30	30	1128	12	1200
4	L4	7.5/4.95	112.5/74.25	45	45	1098	12	1200
5	L5	10/6.6	150/99	60	60	1068	12	1200
6	L6	15/9.9	225/148.5	90	90	1008	12	1200
7	L7	30/19.8	450/297	180	180	828	12	1200
8	L8	60/39.6	900/594	360	360	468	12	1200
9	L9	90/59.4	1350/891	540	540	108	12	1200

- flow rate to ion source: 0.5 ml per minute,
- column temperature: 60°,
- sampler temperature: 18°,
- a gradient programme using the conditions given below,
- injection volume: 20 µl,
- mode: LC,
- detector: MS/MS (triple quadrupole mass spectrometer),

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	97	3
1.5	97	3
4.0	50	50
7.0	25	75
8.1	15	85
9.2	5	95
12.0	5	95
12.1	97	3

MS Conditions

- ionization: Atmospheric pressure chemical ionization (APCI),
- scan settings: see following table

Nitrosamine impurity	Acquisition mode	Polarity	MRM Transitions (m/z): M-2	
			MRM-1[1]	MRM-2
NDMA	MRM	Positive	+75.0 → +43.0	+75.0 → +44.1
NDMA-d6	MRM	Positive	+81.2 → +46.0	+81.2 → +64.1
NDEA	MRM	Positive	+103.1 → +75.1	+103.1 → +47.1
NDEA-d10	MRM	Positive	+113.2 → +34.2	+113.2 → +49.1
NMBA	MRM	Positive	+147.1 → +44.1	+147.1 → +117.1
NMBA-d3	MRM	Positive	+150.1 → +47.1	+150.1 → +120.2
NDBA	MRM	Positive	+159.2 → +41.1	+159.2 → +29.1
NDBA-d18	MRM	Positive	+177.3 → +66.2	+177.3 → +46.2
NEIPA[2]	MRM	Positive	+117.1 → +75.1	+117.1 → +47.2
NDIPA[2]	MRM	Positive	+131.2 → +89.1	+131.1 → +47.1

[1] MRM-1 is used for quantitation.

[2] NDEA-d10 is used as internal standard for NEIPA and NDIPA.

Inject reference solution (c) and generate the peak response ratio of the specified impurity to that of the internal standard versus the concentration standard curve for each nitrosamine impurity under test using the corresponding selected reference solutions (Table 7) and perform the linear regression analysis.

Impurity name	Relative retention time
NDMA	0.20
NMBA	0.31
NDEA	0.46
NEIPA	0.57
NDIPA	0.66
NDBA	1.00

The test is not valid unless the correlation coefficient is not less than 0.99 and Y-intercept is not more than 25 per cent of the response of the medium concentration solution used in standard curve generation.

Inject reference solution (c) and the test solution.

Calculate the concentration in ppm, of each specified nitrosamine impurity in the test solution using the corresponding calibration curve.

Report the nitrosamine impurity concentration in the drug substance in ppm (µg per g).

Method D. Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMPA and NDBA in selected sartans (valsartan, losartan potassium and candesartan cilexetil) by GC-MS/MS (triple-quad).

Internal standard solution. A 50 ng per ml solution of *NDMA: C13-d6 IPRS* in *methylene chloride*.

Test solution. Dissolve 500 mg of the substance under examination into a 10-15 ml glass centrifuge tube. Add 5.0 ml of the internal standard solution. Cap the tube and vortex for 1 minute, and then centrifuge at 4000 rpm for 2.5 minutes. Transfer 2 ml of the bottom methylene chloride layer to a 5-ml syringe fitted with a 0.45-µm nylon filter. Filter 1 ml of sample extract into a 2-ml GC autosampler vial and cap.

Reference solution (a). A solution containing 0.1 µg per ml of, each of, *N-nitrosodimethylamine IPRS, N-nitrosodiethylamine IPRS, N-nitrosoethylisopropylamine IPRS, N-nitrosodiisopropylamine IPRS, N-nitrosomethylphenylamine IPRS* and *N-nitrosodibutylamine IPRS* by mixing appropriate volumes of respective reference standards and diluting with internal standard solution.

Calibration solutions: Depending on the targeted nitrosamine concentration in the sample, prepare a set of 5 consecutive solutions that are used for generating the calibration curve by

following the preparation scheme shown in the below table 8. Volumes may be adjusted to prepare larger quantities of the calibration solutions as needed, maintaining final concentrations of the nitrosamines. For each calibration solution, transfer the designated aliquot of reference solution (a) to the designated volumetric flask, and adjust the volume with the internal standard solution.

Table 8

Calibration solution ID	Standard solution aliquot (µl)	Final volume (ml)	Final Nitrosamine concentration (µg/ml)	Equivalent Nitrosamine concentration (µg/g)
Cal 1	50	10	0.0005	0.005
Cal 2	100	10	0.001	0.010
Cal 3	200	10	0.002	0.020
Cal 4	300	10	0.003	0.030
Cal 5	400	10	0.004	0.040
Cal 6	500	10	0.005	0.050
Cal 7	1000	10	0.010	0.100
Cal 8	1500	10	0.015	0.150

Chromatographic system

- Mode: GC
- a fused-silica column 30 m x 0.25 mm, coated with polyethylene glycol compound (avg. mol. weight about 15,000) (film thickness 1.0 µm) (Such as VF-Wax ms, J&W),
- injector: split/ splitless,
- injector type: splitless with purge,
- purge time: 0.5 minute,
- detector: MS/MS (triple quadrupole mass spectrometer),
- temperature:

Initial Temperature (°)	Temperature Ramp (° per min.)	Final Temperature (°)	Hold Time at Final Temperature (min.)
40	0	40	0.5
40	20	200	0
200	60	250	3

- flow rate. 1.0 ml per minute, using helium as carrier gas (adjustment and verification are necessary for other carrier gases),

MS conditions

- ionization: Electron impact (EI),
- Scan settings: Refer below table

Nitrosamine impurity	Acquisition mode	Polarity	MRM Transitions (m/z) MRM-1[1]	MRM-2
NDMA	MRM	Positive	+74.0 → +44.0	+74.0 → +42.0
NDMA: c13-d6	MRM	Positive	+82.0 → +48.0	
NDEA	MRM	Positive	+102.0 → +85.0	+102.0 → +56.0
NEIPA	MRM	Positive	+116.0 → + 99.0	+ 71.0 → + 56.0
NDIPA	MRM	Positive	+ 130.0 → + 88.0	+ 130.0 → + 42.0
NMPA	MRM	Positive	+ 106.0 → + 77.0	+77.0 → +51.0
NDBA	MRM	Positive	+158.0 → +99.0	+ 84.0 → +56.0

[1] MRM-1 is used for quantitation.

MS1 and MS2 resolution: Q1: normal; Q3: wide (1.5),

- Minimum window: 1 minute,
- Emission current: 50 µA,
- Injector temperature: 250°,
- Transfer line to MS detector temperature: 250°,
- Ionization source temperature: 250°,
- Injection volume: 2 µl.

Inject calibration solutions.

Impurity name	Relative retention time
NDMA	0.73
NDEA	0.78
NEIPA	0.81
NDIPA	0.82
NDBA	0.99
NMPA	1.00

Generate the peak response ratio of the specified impurity to that of the internal standard versus concentration standard curve for each nitrosamine impurity under test using the corresponding Calibration solutions and perform the linear regression analysis.

The test is not valid unless the correlation coefficient is not less than 0.98 and signal-to-noise ratio is not less than 10 for the impurity peak of the lowest concentration calibration solutions used in the calibration curve.

Inject calibration solutions and the test solution.

Calculate the concentration, in ppm, of each specified nitrosamine impurity in the sample solution using the corresponding calibration curve.

Method E. LC-HRMS method for determination of NDMA impurity in Ranitidine drug substance and drug products.

N-nitroso-*di*-methylamine (NDMA) impurity is separated from ranitidine by reverse phase chromatography and is detected by a high-resolution and high-mass accuracy (HRAM) mass spectrometer. High sensitivity detection is achieved by monitoring the accurate *m/z* value of the protonated impurity ion. Quantitation is performed by comparing the peak area of the NDMA impurity in extracted ion chromatogram of the samples to the peak area of the NDMA reference standard in an external calibration standard.

Blank. Methanol

Test solution

For drug substance. Weigh accurately 120 mg of substance under examination into a 15 ml glass centrifuge tube. Add 4.0 ml of *methanol* and mix the solution using a vortex mixer until dissolved.

For drug product. Shake a quantity of the powdered tablets containing 0.15 g of drug substance with 5 ml of *methanol,* transfer into a centrifuge tube, vortex for about a minute and shake the solution for 40 minutes using a mechanical wrist action shaker.

After extraction, centrifuge the sample for 15 minutes at 4500 rpm. Filter the supernatant using a 0.22 µm PVDF syringe filter, discard the first 1 ml and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Reference solution (a). A 100 ng per ml solution of *N-Nitrosodimethylamine IPRS* in *methanol.*

Reference solution (b). Dilute 1.0 ml of the reference solution (a) to 50 ml with *methanol.* Prepare the solution fresh daily (2.0 ng/ml).

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (3 µm) (Such as Ace C18-AR, 100 Å),
- mobile phase: A. 0.1 per cent v/v *formic acid* in *water,* B. 0.1 per cent v/v *formic acid* in *acetonitrile,*
- flow rate: 0.5 ml per minute,
- column temperature: 30°,
- sample temperature: 4° – 8°,
- needle wash: 80:20 *methanol* : *water* with 0.1 per cent v/v *formic acid,*
- a gradient programme using the conditions given below,
- detector: LC/MS (mass spectrometer),
- injection volume: 3 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
1	95	5
3	80	20
7	0	100
9	0	100
9.1	95	5
14	95	5

MS conditions

- Ion source settings (See below table)

NOTE — Ion source parameters can be adjusted to achieve the desired sensitivity.

Sheath Gas Flow Rate	55 arbitrary units
Aux Gas Flow Rate	15 arbitrary units
Sweep Gas Flow Rate	0 units
Spray Voltage	3.5 kV
Capillary Temp.	400°
Aux Gas Heater Temp.	350°

- Scan settings: see following table

NOTE — 1. The scan start-end time should be adjusted for the user's HPLC system since the retention time of the NDMA impurity may vary between different HPLC systems,

2. The divert valve can be used to divert the eluent to waste when a scan is not performed.

NDMA Impurity

Scan Type	PRM
Polarity	Positive
Scan Start -End (min)	0 -3.2
m/z Isolated for PRM	75.0553
NCE	80
Isolation window	1.5 m/z
Scan range	50 – 95 *m/z*
Microscans	3
Resolution	35,000
AGC target	2e5
Maximum IT	100 ms

Inject the blank and reference solution (b). The test is not valid unless the relative standard deviation and cumulative relative standard deviation for six consecutive injections is not more 10 per cent and 15 per cent, respectively in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Data processing

- NDMA Peak areas in the extracted ion chromatograms (EIC) with an m/z tolerance of ±15 ppm are used for quantitation. The NDMA m/z value to be extracted is listed in below table

Nitrosamine Impurity	NDMA
m/z to be extracted	75.0553

- The retention time difference of the NDMA impurity in the analyzed samples should not be more than 2 per cent of the retention time of the corresponding NDMA peak in the reference solution.

Calculate the nitrosamine impurity (NDMA) content in the drug substance and drug product in ppm (µg per g).

Method F. LC-HRMS method for the determination of NDMA in Metformin drug substance and drug products.

N-nitroso-*di*-methylamine (NDMA) impurity is separated from metformin by reverse phase chromatography and is detected by a high-resolution and high-mass accuracy (HRAM) mass spectrometer. High sensitivity detection is achieved by monitoring the accurate *m/z* value of the protonated NDMA ion. Quantitation is performed by comparing the peak area of the NDMA impurity in extracted ion chromatogram of the samples to the peak area of the NDMA reference standard in external calibration standard.

Blank. Methanol

Test solution

For drug substance. Weigh accurately 500 mg of substance under examination into a 15 ml glass centrifuge tube. Add 5.0 ml of *methanol* and mix the solution using a vortex mixer and shake the solution for 40 minutes using a mechanical wrist action shaker.

For drug product. Shake a quantity of the powdered tablets containing 0.500 g of drug substance with 5 ml of *methanol,* transfer into a centrifuge tube, vortex for about a minute and shake the solution for 40 minutes using a mechanical wrist action shaker.

After extraction, centrifuge the sample for 15 minutes at 4500 rpm. Filter the supernatant using a 0.22 µm PVDF syringe filter, discard the first 1 ml and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Reference solution (a). A 100 ng per ml solution of *N-Nitrosodimethylamine IPRS* in *methanol.*

Reference solution (b). Dilute 3.0 ml of the reference solution (a) to 100 ml with *methanol.* Prepare the solution fresh daily (3.0 ng/ml).

Chromatographic system

- a stainless steel column 15 cm x 3 mm, packed with octadecylsilane bonded to porous silica (2.5 µm) (Such as X Select CSH C18 XP, 130Å),
- column temperature: 30°,
- sample temperature: 20°,
- mobile phase: A. 0.1 per cent v/v *formic acid* in *water,*
  B. 0.1 per cent v/v *formic acid* in *methanol,*
- flow rate: 0.3 ml per minute,
- needle wash: 80:20 *methanol* : *water* with 0.1 per cent v/v *formic acid,*
- a gradient programme using the conditions given below,
- detector: LC/MS (mass spectrometer),
- injection volume: 3 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5.0	90	10
6.0	10	90
9.0	10	90
9.1	90	10
14.0	90	10

MS Conditions

- Ion Source Settings (See below table)

NOTE — Ion source parameters can be adjusted to achieve the desired sensitivity.

Sheath Gas Flow Rate	50 arbitrary units
Aux Gas Flow Rate	15 arbitrary units
Sweep Gas Flow Rate	0 units
Spray Voltage	3.5 kV
Capillary Temp.	350°
Aux Gas Heater Temp.	350°

- Scan settings: see following table

NOTE — 1. The scan start-end time should be adjusted for the user's HPLC system since the retention time of the NDMA impurity may vary between different HPLC systems.

2. The divert valve can be used to divert the eluent to waste when a scan is not performed.

NDMA Impurity

Scan Type	PRM
Polarity	Positive
Scan Start -End (min)	3.2-5.0
m/z Isolated for PRM	75.0553
NCE	80
Isolation Window	1.5 m/z
Microscans	3
Resolution	35,000
AGC target	2e5
Maximum IT	100 ms

Inject the blank and reference solution (b). The test is not valid unless the relative standard deviation and cumulative relative standard deviation for six consecutive injections is not more 10 per cent and 15 per cent respectively in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Data processing

- NDMA Peak areas in the extracted ion chromatograms (EIC) with an m/z tolerance of ±15 ppm are used for quantitation. The NDMA m/z value to be extracted is listed in below table

Nitrosamine Impurity	NDMA
m/z to be extracted	75.0553

- The retention time difference of the NDMA impurity in the analyzed samples should not be more than 2 per cent of the retention time of the corresponding NDMA peak in the reference standard solution.

Calculate the nitrosamine impurity (NDMA) content in the drug substance and drug product in ppm (µg per g).

Additional Sources of Information

Several test procedures have been developed for the specific testing of nitrosamines in sartans and/or other official articles based on different scientific principles and are publicly available from many regulatory agencies. These can be used as alternative procedures and must be validated under actual use for the respective performance characteristics recommended.

6. PRIMARY PACKAGES FOR PHARMACEUTICAL ARTICLES

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6.1. Introduction

Pharmaceutical articles are delivered to the intended consumers in a variety of packages.

A package for pharmaceutical articles is a form that is intended to contain, protect or preserve the contents throughout the shelf life; aid in the safe, appropriate transportation and distribution; and to inform the user about the pharmaceutical article. A package comprises a container that usually has a closure (e.g. cap, lid, dispensing system) and a label (e.g. a separate label or printed matter). The closure may or may not have a liner.

A package is also conventionally referred to as a container-closure system.

*NOTE 1 — **Scope***

This section covers the primary package i.e. the materials that come in direct contact with pharmaceutical contents. Secondary and tertiary packageare not addressed in this pharmacopoeia.

This chapter deals with the specific requirements, guidance and information on containers used for packaging of pharmaceutical articles. The materials that are used in the manufacture of containers, particularly plastic containers, the raw materials and the additives used and the formulations employed should be agreed with the users of the containers.

A container-closure system for pharmaceutical article is intended to contain a drug substance or drug product with which it is, or may be in direct contact.

A container-closure system for Pharmaceutical article must be chosen with care and after taking into consideration the nature of the articles and the likely effects of transportation and storage, even for short periods of time.

A container-closure system for Pharmaceutical article should be designed so that the contents may be removed in a manner suitable for the intended use of the article in it. It should also provide an adequate degree of protection, minimise the loss of constituents and should not interact physically or chemically with the contents in a way that will alter their quality to an extent beyond the limits given in the individual monograph, or present a risk of toxicity.

The choice of a container-closure system for Pharmaceutical article is also governed by the likely period of storage of the article during which its quality will not be compromised to a degree where it will be unfit for use. Under the heading Storage, the label indicates the measures to be taken to protect the article from contamination and deterioration during its entire shelf-life. Technical Specifications for the package to be used for any article have not been given but in certain cases, the type of package that is recommended is stated in terms that have the following meanings.

6.1.1. Terminology

a. ***Package***. A package comprises a container that usually has a closure (e.g. cap, lid, dispensing system) and a label (e.g. a separate label or printed matter). The closure may or may not have a liner.

   A package is also conventionally referred to as a container-closure system.

b. ***Well-closed Package***. A container-closure system for a Pharmaceutical article that protects the contents from extraneous solids and liquids and loss of the article in process of handling, shipment, storage and distribution.

c. ***Airtight or Tightly-closed Package***. A container-closure system for a Pharmaceutical article that protects the contents from contamination by extraneous solids, liquids, or vapours, loss or deterioration of the article from effervescence, deliquescence or evaporation during handling, shipment, storage and distribution. A tightly-closed container-closure system for a Pharmaceutical article must be capable of being tightly re-closed after use.

d. ***Hermetically Sealed Package***. A container-closure system for a Pharmaceutical article that is impervious to air or any other gas under normal conditions of handling, shipment, storage and distribution, e.g. sealed glass ampoule, gas cylinder etc. A hermetically sealed container-closure system must beused for a single dose.

e. ***Light-resistant Package***. A container-closure system for a Pharmaceutical article that protects the contents from the effects of actinic light by virtue of the specific properties of the material of which it is made. Alternatively, a clear and colourless or a translucent container may be made light-resistant by means of an opaque (light-resistant) covering and/or in such cases, the label on the container should bear a statement that the opaque covering or storage in dark place is needed until the contents have been used up.

f. ***Single-dose Package***. A container-closure system for Pharmaceutical articlethat holds a quantity of the preparation intended for total or partial use as a single administration.

g. ***Multi-dose Package***. A container-closure system for a Pharmaceutical article that holds a quantity of the preparation suitable for two or more doses.

h. ***Sealed Package***. A container-closure system for Pharmaceutical article closed by fusion of the material of the container.

i. ***Tamper-evident Package***. A container-closure system for a Pharmaceutical article fitted with a device or mechanism that reveals irreversibly whether the container has been opened.

The user of the container-closure system must obtain an assurance from the supplier that its composition does not vary from batch to batch and that it is identical to that used during compatibility testing.

When the user is informed of changes in the composition, compatibility testing and biological testing must be repeated, totally or partly depending on the nature of the changes.

A container-closure system for a Pharmaceutical article is available in different forms. Details of the commonly used components are given below:

6.2. CONTAINERS

Containers for pharmaceutical articles are made of a variety of materials such as different types of plastics, glass, metal and their combination. This section covers the guideline for each type of these container materials.

6.2.1. Plastic Containers

The commonly used plastic resins conforming to Indian Standards for the manufacture of plastic container and closures are Polyethylene terephthalate (PET) (IS 12252), Polyethylene (IS 10146), Polyvinyl Chloride (IS 10151), Polypropylene (IS 10910), Cyclic Olefins, Polyamides [nylon 6 (IS 12247)], Polycarbonate (IS 14971), Poly (ethylene-vinyl acetate) (IS 13601). However, if the manufacturer of Pharmaceutical articles intends to use plastics made from polymers other than listed above, it should be justified and authorised by appropriate competent authority.

The plastic identification codes as mentioned in IS 14534 may be visibly marked on the plastic containers and/ or the label, wherever possible.

The plastic containers should be manufactured from materials that do not include in their composition any substances that can be extracted by the pharmaceutical contents in such quantities that alter the efficacy or stability of the product or present a toxic hazard. Recycling of excess material (shavings, dust, etc. from the container manufacturing plant) of well-defined nature and proportions may be permitted after appropriate validation. However, the use of post-manufactured material is strictly prohibited. Re-use of earlier-filled bottles is strictly prohibited.

For general information, a summary of the manufacture and usage of plastic containers and closures is provided in the Table 1.

Table 1 - Overview of the life cycle (stages) of Plastic Materials for Pharmacopoeial Articles

Stakeholder	Activity Involved	Governing Regulations in force	Examples
Plastic Resin Manufacturers	**Monomers** (Raw material) ↓ conversion into ↓ **Polymer** (Plastic Resin)	BIS Standards on composition of various packaging polymers (plastic resins)	Polyethylene terephthalate (PET) = IS 12252 Polyethylene (PE) = IS 10146 Polyvinyl chloride (PVC) = IS 10151 Polypropylene (PP) = IS 10910 Polyamides [nylon 6] = IS 12247 Polycarbonate = IS 14971 Poly (ethylene-vinyl acetate) = IS 13601
Manufacturers of Packaging components (Convertors)	Addition of Colourant/ Additives to Resin if neededand converting the mixture into shaped articles (Plastics Articles)	BIS Standard for Resin Identification Codes IS - 14534	Bottles, Containers, Tubes, Caps, Closures and other forms of Packages **Identification Codes** Code No.1 = PET Code No.2 = HDPE (High Density PE) Code No.3 = V (PVC, Polyvinyl Chloride) Code No.4 = LDPE (Low Density PE) Code No.5 = PPCode No.6 = PS (Polystyrene) Code No.7 = Other plastics
Manufacturer of Pharmacopoeial Articles (Users)	Filling of Pharmaceutical Formulations (Packaged Products)	The Drugs and Cosmetics Act, 1940 The Drugs and Cosmetics Rules, 1945	Substances, Preparations, Articles
Consumer	Consumption/Usage	—	Patients, Health care chain (Doctors, Hospitals)
All stakeholders	Disposal	Plastic Waste Management Rules, 2016	Various responsibilities on Manufacturers, Local Bodies, Citizens, etc.

The selection of a suitable plastic container should be based on a knowledge - obtained from the supplier - of the composition of the plastic (e.g. raw materials, colourants and additives) so that potential hazards can be assessed.

The plastic container chosen for any particular pharmaceutical product should be such that the ingredients of the product in contact with the container do not significantly interact or migrate into or through the plastic, to a level that affects the quality of the product.

Test on Container Material

Type samples (specimen) of the intended container should be packed with the product and tested under conditions that reproduce those that would be encountered in use. These tests should include examination of the product to ensure absence of any sensory, chemical or physical change, an assessment of changes in the quality of contents due to permeability of the plastic, detection of changes in pH, an assessment of the effects of light, chemical tests and where necessary, biological tests. Containers from bulk production should conform to the type sample (specimen) in every respect. It should be ensured that there is no change in the composition or any change in the manufacturing method used by the manufacturer and more importantly, that no use is made of post-consumer recycled material. It must be emphasized that changes in the composition of the plastic or reworking or inadequate control of processing can bring about changes which may invalidate the results of type testing. Samples from production should be tested to ensure conformance to type samples (specimen) and test schedules should be designed to check deviations from the characteristic of the type sample (specimen).

The biological and chemical tests described below are intended for plastic containers in which pharmaceutical formulations are packaged. It should be appreciated that these tests by themselves are not sufficient to establish safety or suitability of the plastic containers for the preparations and it is necessary to consider the results of the tests in conjunction with the information given above. Specification should be agreed with the container manufacturer and should be revised if the composition of the plastic or the ingredient quality is altered or the processing treatment is changed.

*NOTE 2 — **Applicability of Biological Tests for Plastic Packages***

a) Packages used for non-parenteral dosage forms do not require any biological testing.

b) Packages used forparenteral dosage forms require *in vitro* testing (2.2.23).

c) Packages that meet the requirements of the *in vitro* tests are not required to undergo any further *in vivo* testing (2.2.24).

d) Packages that do not meet the requirements of the *in vitro* tests where applicable are required to undergo further *in vivo* testing.

Packages that do not meet the requirements of the biological reactivity tests [(2.2.23) and (2.2.24), if appropriate] are not suitable as packages for pharmaceutical use.

NOTE 3 —**Responsibility**

It shall be the responsibility of the user/manufacturer to ensure the compatibility of the pharmaceutical articles with the package and stability of the product in a package.

Towards this:

a) The user/manufacturer shall have in place a system of quality assurance with the supplier / convertor to ensure compliance of the components of the package with the requirement stated in this chapter at all times.

b) The initial protocol for approving the vendor and its packaging materials shall involve the entire test regimen as detailed in subsequent relevant sections of this chapter. Subsequently for every lot of the packaging materials only the following tests shall be carried out by the user (pharmaceutical manufacturers) :

   i. Identification by FTIR
   ii. Visual Inspection
   iii. Leakage
   iv. All tests related to appearance, absorbance, acidity and alkalinity

6.2.1.1. Plastic Containers for Parenteral Preparations

General Requirements

Material. Plastic containers for parenteral preparations are manufactured from one or more polymers. The polymers most commonly used are polyethylene, polypropylene and polyvinyl chloride. Only virgin plastic material, which is practically odourless, is used in the manufacture of the containers. Add additives such as antioxidants, lubricants, plasticisers, stabilisers, etc. may be used but no pigment may be used for purposes of colouring.

Characteristics. The containers may be bags or bottles. They have a site suitable for the attachment of an infusion set designed to ensure a secure connection. They may have a site that allows an injection to be made at the time of use. They usually have a part that allows them to be suspended and withstand the stresses during use. Although it may not be feasible to include parameters for construction and design of containers in terms of size, shape and weight, for example those meant for large volume parenterals (LVP), of different materials and made on different machines, both manufactured indigenously and internationally, involved in the production of such plastic containers, nevertheless the integrity of neck

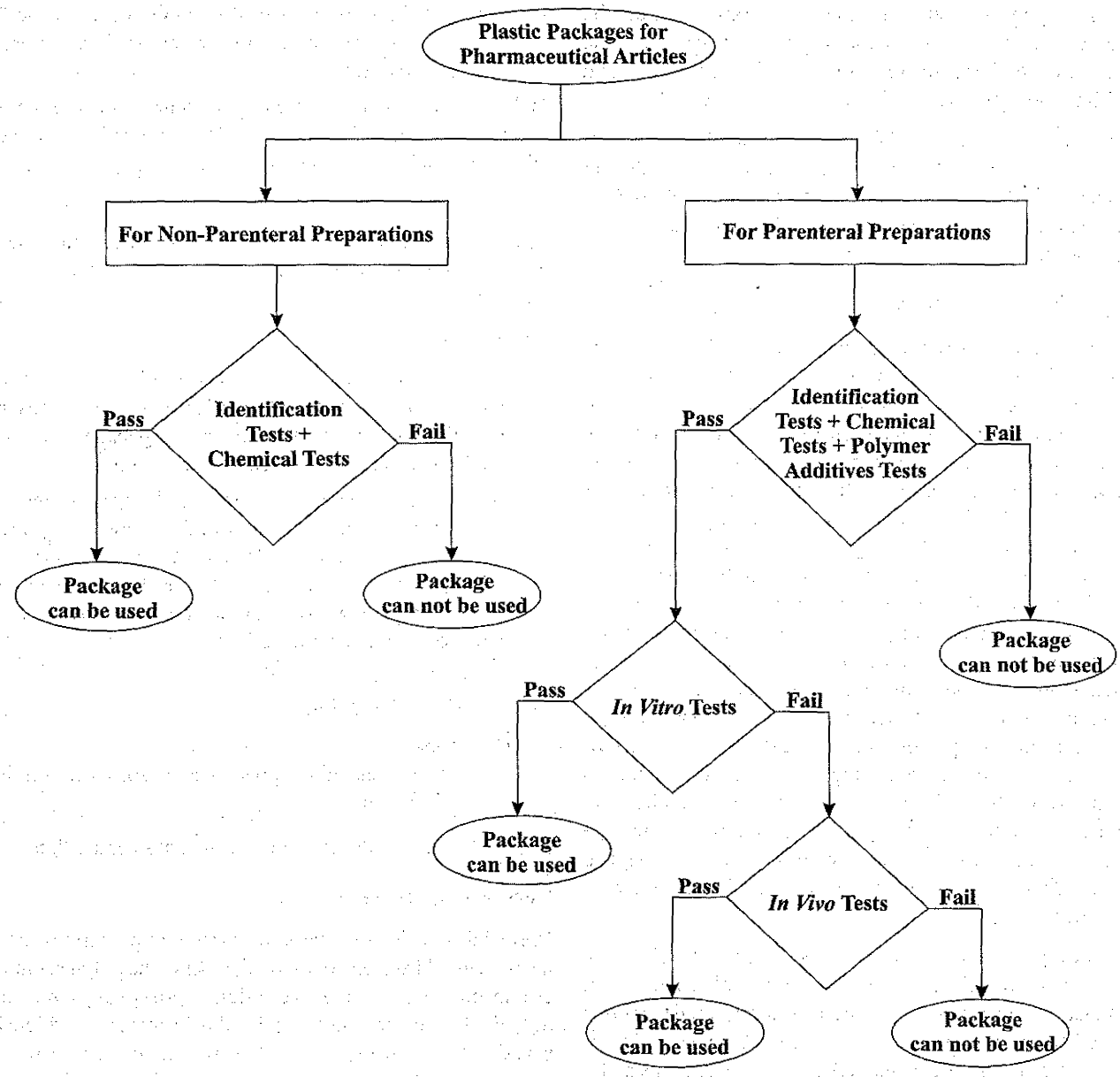


Fig. - Approval Philosophy-Plastics for Pharmaceutical Packaging

and shoulders of the containers should be suitably and appropriately strengthened and it shall be the responsibility of such LVP manufacturers to ensure that the containers withstand the stresses and rigors of transportation and packaging. The containers must withstand the sterilisation conditions to which they will be subjected. The design of the container and the method of sterilisation chosen are such that all parts of the containers that may be in contact with the infusion are sterilised. The containers are impermeable to micro-organisms after closure. The containers are such that after filling them, they are resistant to damage from accidental freezing which may occur during transport of the final preparation. The containers are and remain sufficiently transparent to allow the appearance of the contents to be examined at any time, unless otherwise justified and authorised.

Labelling. The label accompanying a batch of empty containers states (1) the name and address of the manufacturer; (2) a batch number which enables tracing the history of the container and of the plastic material of which it is manufactured.

Visual inspection. The empty containers display no defects that may lead to leakage and the filled and closed container shows no leakage.

Tests on Containers

Leakage test. Fill ten containers with water, fit them with the intended closures and keep them inverted at room temperature for 24 hours.

There are no signs of leakage from any container.

Collapsibility test. This test is applicable to containers which are to be squeezed in order to remove the contents. A container, by collapsing inward during use, yields at least 90 per cent of its nominal contents at the required rate of flow at ambient temperature.

*NOTE 4 — **Testing of Secondary Packages - Special Case***

For satisfactory storage of some preparations, the container needs to be enclosed in a protective envelope. In such cases, the initial evaluation of storage is carried out using the container enclosed in the envelope.

*NOTE 5 — **Additional requirements on Plastic containers for Parenteral Preparations***

For Plastic Containers for Parenteral Preparations

a) Other suitable means of ensuring package integrity may also be used.

b) All considerations covered in details in the Plastic Containers for Non-parenteral Preparations (6.2.1.2) shall apply.

Tests using Special Solution "S"

Preparation of Special Solution "S"

Fill a container under examination to its nominal capacity with water and close it, using the usual means of closure; otherwise closed using a suitable sheet / foil of aluminium. Heat in an autoclave so that a temperature of 121± 2° is reached within 20 to 30 minutes and maintain at this temperature for 30 minutes. If heating at 121° leads to deterioration/deformation of the container, heat at 100° for 2 hours.

NOTE — Use solution S within 4 hours of its preparation.

Blank. Prepare a blank by heating water in a borosilicate-glass flask closed by an aluminium foil at the temperature and for the time used for the preparation of solution S.

Tests Using Solution "S"

Appearance of solution S. Solution S is clear (2.4.1) and is colourless (2.4.1).

Acidity or Alkalinity. To a volume of solution S corresponding to 4 per cent of the nominal capacity of the container add 0.1 ml of *phenolphthalein solution*. The solution is colourless.

Add 0.4 ml of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 ml of *0.01 M hydrochloric acid* and 0.1 ml of *methyl red solution*. The solution is orange-red or red.

Light absorption. The light absorption in the range 230 nm to 360 nm of solution S using a blank prepared as described under Solution S is not more than 0.20 (2.4.7).

Reducing substances. To 20.0 ml of solution S add 1 ml of *dilute sulphuric acid* and 20.0 ml of *0.002 M potassium permanganate*. Boil for 3 minutes. Cool immediately. Add 1 g of *potassium iodide* and titrate immediately with *0.01 M sodium thiosulphate*, using 0.25 ml of *starch solution* as indicator.

Carry out a titration using 20.0 ml of the blank prepared as described under Solution S.

The difference between the titration volumes is not more than 1.5 ml.

Transparency. Fill the containerpreviously used for the preparation of solution Sto its nominal capacity with a 1 in 200 dilution of the standard suspension (2.4.1) when made from polyethylene or polypropylene. For containers made of other plastics, use a 1 in 400 dilution.

The cloudiness of the suspensionis perceptible when viewed through the container and compared with a similar container filled with *water* (2.4.1).

6.2.1.1.1. Sterile Plastic Containers for Blood and Blood Components

Plastic containers for the collection, storage, processing and administration of blood and its components are manufactured from one or more polymers, if necessary with additives. The composition and the conditions for manufacture of the containers are approved/registered by the appropriate competent authorities in accordance with the relevant national legislation and international agreements.

When the composition of the materials of the different parts of the containers corresponds to the appropriate specifications, their quality is controlled by the methods indicated in the specifications, described under Plastic Containers for Parenteral Preparations (6.2.1.1).

Materials other than those described in this Pharmacopoeia may be used provided that their composition is authorised by the Licensing Authority and that the containers manufactured from them comply with the requirements prescribed for Sterile Plastic Containers for Blood and Blood Components.

In normal conditions of use the plastic materials do not release monomers, or other substances, in amounts likely to be harmful and do not lead to any abnormal modifications of the blood. The containers may contain anticoagulant solutions, depending on their intended use, and are supplied sterile.

Each container is fitted with attachments suitable for the intended use. The container may be in the form of a single unit or the collecting container may be connected by one or more tubes to one or more secondary containers to allow separation of the blood components to be effected within a closed system.

The outlets are of a shape and size allowing for adequate connection of the container with the blood-giving equipment. The protective coverings on the phlebotomicneedle and on the appendages should be such as to ensure the maintenance of sterility. They should be easily removable but should be tamper-proof.

The capacity of the containers is related to the nominal capacity prescribed by the national authorities and to the appropriate volume of anticoagulant solution. The nominal capacity is the volume of blood to be collected in the container. The containers are of a shape such that when filled they may be centrifuged.

The containers are fitted with a suitable device/appendage for continuous flow which does not hinder the collection, storage, processing or administration of the blood.

The containers are enclosed in sealed, protective envelopes (See NOTE 4).

Description. The container is sufficiently transparent to allow adequate visual examination of its contents before and after the taking of the blood and is sufficiently flexible to offer minimum resistance during filling and emptying under normal conditions of use. The container contains not more than 5.0 ml of air.

Tests

Solution S1. Fill the container with 100.0 ml of 0.9 per cent w/v solution of sodium chloride. Close the container and heat it in an autoclave so that the contents are maintained at 110° for 30 minutes.

If the container under examination contains an anticoagulant solution, first empty it, rinse the container with 250.0 ml of *water for injections* at 20 ± 1° and discard the rinsings.

Solution S2. Introduce into the container a volume of *water for injections* corresponding to the intended volume of anticoagulant solution. Close the container and heat it in an autoclave so that the contents are maintained at 110° for 30 minutes. After cooling, add sufficient water for injections to fill the container to its nominal capacity.

If the container under examination contains an anticoagulant solution, first empty it and rinse it as indicated above.

Resistance to centrifugation. Introduce into the container a volume of *water,* acidified by the addition of 1 ml of *dilute hydrochloric acid,* sufficient to fill it to its nominal capacity. Envelop the container with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue* reagent or other suitable indicator and then dried. Centrifuge at 5000 rpm for 10 minutes.

No leakage is perceptible on the indicator paper and no permanent distortion occurs.

Resistance to stretch. Introduce into the container a volume of *water,* acidified by the addition of 1 ml of *dilute hydrochloric acid,* sufficient to fill it to its nominal capacity. Send the container by the sending device at the opposite end from the blood-taking tube and apply along the axis of this tube an immediate force of 20 N (2.05 kgf). Maintain the traction for 5 seconds. Repeat the test with the force applied to each of the parts for filling and emptying.

No break and no deterioration occurs.

Leakage. Place the container that has been submitted to the stretch test between two plates covered with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue* reagent or other suitable indicator and then dried. Progressively apply force to the plates to press the container so that its internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) reaches 67 kPa within 1 minute. Maintain the pressure for 10 minutes.

No signs of leakage are detectable on the indicator paper or at any point of attachment (seals, joints, etc.).

Vapour permeability. For a container containing an anticoagulant solution, fill with a volume of 0.9 per cent w/v solution of sodium chlorideequal to the volume of blood for which the container is intended.

For an empty container, fill with the same mixture of anticoagulant solution and 0.9 per cent w/v solution of sodium chloride. Close the container, weigh it and store it at 5 ± 1° in an atmosphere with a relative humidity of 50 ± 5 per cent for 21 days.

At the end of this period the loss in weight is not more than 1 per cent.

Emptying under pressure. Fill the container with a volume of *water* at 5 ± 1° equal to the nominal capacity. Attach a transfusion set without an intravenous cannula to one of the connectors. Compress the container so as to maintain throughout the emptying an internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) of 40 kPa.

The container empties in less than 2 minutes.

Speed of filling. Attach the container by means of the blood-taking tube fitted with the needle to a reservoir containing a suitable solution having a viscosity equal to that of blood, such as a 33.5 per cent w/v solution of *sucrose* at 37°. Maintain the internal pressure of the reservoir (i.e. the difference between the applied pressure and atmospheric pressure) at 9.3 kPa with the base of the reservoir and the upper part of the container at the same level. The volume of liquid which flows into the container in 8 minutes is not less than the nominal capacity of the container.

Resistance to temperature variations. Place the container in a suitable chamber having an initial temperature of 20° to 23°. Cool it rapidly in a deep-freezer to –80° and maintain it at this temperature for 24 hours. Raise the temperature to 50° and maintain for 12 hours. Allow to cool to room temperature.

The container complies with the tests for Resistance to centrifugation, Resistance to stretch, Leakage, Vapour permeability, Emptyingunder pressure and Speed of filling described above.

Transparency. Fill the empty container with a volume equal to its nominal capacity of the standard suspension (2.4.1), diluted so as to have an absorbance at 640 nm of 0.37 to 0.43 (dilution factor about 1 in 16) (2.4.7).

The cloudiness of the suspension must be perceptible when viewed through the bag, as compared with a similar container filled with *water*.

Extractable matter. Tests are carried out by methods designed to simulate as far as possible the conditions of contact between the container and its contents which occur in conditions of use.

The conditions of contact and the tests to be carried out on the elutes are described, according to the nature of the constituent materials, in the particular requirements for each type of container.

Haemolytic effects in buffered systems

Stock buffer solution. Dissolve 90.0 g of *sodium chloride*, 34.6 g of *sodium phosphate* and 2.4 g of *sodium dihydrogen phosphate dihydrate* in *water* and dilute to 1000 ml with the same solvent. Prepare three buffer solutions as follows

Buffer solution Ao. To 30.0 ml of stock buffer solution add 10.0 ml of *water*.

Buffer solution Bo. To 30.0 ml of stock buffer solution add 20.0 ml of *water*.

Buffer solution Co. To 15.0 ml of stock buffer solution add 85.0 ml of *water*.

Introduce 1.4 ml of solution S2 into each of three centrifuge tubes. To tube I add 0.1 ml of buffer solution Ao, to tube II add 0.1 ml of buffer solution Bo and to tube III add 0.1 ml of buffer solution Co. To each tube add 0.02 ml of fresh, heparinised human blood, mix well and warm on a water-bath at 30±1° for 40 minutes. Use blood collected less than 3 hours previously or blood collected into either an Anticoagulant Citrate Phosphate Dextrose Solution (CPD solution) or Anticoagulant Citrate phosphate Dextrose Adenine Solution (CPDA solution) less than 24 hours previously.

Prepare further three solutions as follows:

- 3.0 ml of buffer solution Ao and 12.0 ml of *water* (solution A1),
- 4.0 ml of buffer solution Bo and 11.0 ml of *water* (solution B1),
- 4.75 ml of buffer solution Bo and 10.25 ml of *water* (solution C1),

To tubes I, II and III add, respectively, 1.5 ml of solution A1, 1.5 ml of solution B1 and 1.5 ml of solution C1. At the same time and in the same manner, prepare three other tubes, replacing solution S2 by *water*.

Centrifuge simultaneously the tubes to be examined and the control tubes at exactly 2500 g in the same horizontal centrifuge for 5 minutes. After centrifuging, measure the absorbance of the liquids at about 540nm (2.4.7), using the stock buffer solution as blank. Calculate the haemolytic value as a percentage from the expression

$$\frac{A_{exp}}{A_{100}} \times 100$$

where, A_{100} = absorbance of tube III,

A_{exp} = absorbance of tube I or II or of the corresponding control tubes.

The solution in tube I give a haemolytic value not greater than 10 per cent and the haemolytic value of the solution in tube II does not differ by more than 10 per cent from that of the corresponding control tube.

Sterility. Introduce aseptically into the container 100.0 ml of 0.9 per cent w/v solution of sodium chlorideand shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents of the container through a membrane filter. Complete the test as described under Method of Test for aqueous solutions (2.2.11), paragraph 2, beginning at the words 'After filtration, ...'.

Pyrogens. Solution S1complies with the test for pyrogens (2.2.8). Inject 10.0 ml of the solution per kilogram of the rabbit's weight.

Abnormal toxicity. Solution S1 complies with the general test for abnormal toxicity (2.2.1). Inject 0.5 ml of the solution into each mouse.

Containers and Closures. Sterile plastic containers for human blood and blood components are packed in protective tamper-evident envelopes. On removal from its protective envelope thecontainer shows no leakage and no growth of micro-organisms. The protective envelope is sufficiently robust to withstand normal handling.

The protective envelope is sealed in such a manner that it cannot be opened and re-closed without leaving visible traces that the seal has been broken.

Labelling. The label states that once withdrawn from its protective envelope, the content must be used within 10 days.

A part of the label is reserved for the information required concerning the blood or blood components for which the container is intended to be used.

The ink, or other substance used to print the labels or the writing must not diffuse into the plastic material of the container and must remain legible up to the time of use.

6.2.1.1.2. Sterile PVC (Polyvinyl chloride) Containers for Blood and Blood Components

Sterile PVC (Polyvinyl chloride) containers for blood and blood components should meet the requirements described under the introductory part of section 6.2.1.of chapter 6.2. They also comply with the tests described under Sterile Plastic Containers for Blood and Blood Components and with the following additional tests.

Acidity or Alkalinity. Introduce into the container a volume of *water* corresponding to the intended volume of anticoagulant solution. Close the container and heat in an autoclave so that the contents are maintained at 110°C for 30 minutes. Cool and add sufficient *water* to fill the container to its nominal capacity (solution A). To a volume of solution A corresponding to 4 per cent of the nominal capacity of the container add 0.1ml of *phenolphthalein solution;* the solution remains colourless.

Add 0.4 ml of *0.1 M sodium hydroxide;* the solution is pink. Add 0.8 ml of *0.01 M hydrochloric acid* and 0.1 ml of *methyl red solution;* the solution is orange-red or red.

Light absorption. Heat *water* in a round bottom flask in an autoclave at 110° for 30 minutes (solution B). Measure the light absorption of solution A in the range 230 nm to 360 nm using solution B as blank.

The absorbance is not more than 0.30 at any wavelength from 230 nm to 250 nm and not more than 0.10 at any wavelength from 251 nm to 360 nm (2.4.7).

Ammonium. Dilute 5.0 ml of solution A to 14.0 ml with *water* in a test-tube, if necessary make alkaline with *2 M sodium hydroxide* and dilute further to 15.0 ml with *water*. Add 0.3 ml of *alkaline potassium mercuric-iodide solution*, stopper the tube, mix and allow to stand for 5 minutes.

When viewed vertically, any yellow colour produced is not more intense than that obtained by treating a mixture of 10.0 ml of *ammonium standard solution (1 ppm NH4)* and 5.0 ml of *water* in the samemanner (2 ppm).

Chlorides. 15.0 ml of solution A complies with the limit test for chlorides (2.3.12). Prepare the standard using a mixture of 1.2 ml of *chloride standard solution (5ppm Cl)* and 13.8 ml of *water (0.4ppm)*.

Extractable di(2-ethylhexyl)phthalate

Extraction solvent. Ethanol diluted with *water* to have a relative density of 0.9389 to 0.9395 (2.4.29), measured with a pycnometer.

Stock solution. Dissolve 0.1 g of *di(2-ethylhexyl)phthalate* in the extraction solvent and dilute to 100.0 ml with the same solvent.

Standard solutions

(a) Dilute 20.0 ml of stock solution to 100.0 ml with extraction solvent.

(b) Dilute 10.0 ml of stock solution to 100.0 ml with extraction solvent.

(c) Dilute 5.0 ml of stock solution to 100.0 ml with extraction solvent.

(d) Dilute 2.0 ml of stock solution to 100.0 ml with extraction solvent.

(e) Dilute 1.0 ml of stock solution to 100.0 ml with extraction solvent.

Measure the absorbance of the standard solutions at the maximum at about 272 nm, using the extraction solvent as blank and plot a curve of absorbance against the concentration of di(2-ethylhexyl)phthalate (2.4.7).

Extraction procedure. Using the donor tubing and the needle or adaptor, fill the empty container with a volume equal to half the nominal volume with the extraction solvent, previously heated to 37° in a well-stoppered flask. Expel the air completely from the container and seal the donor tube. Immerse the filled container in a horizontal position in a water-bath maintained at 37 ± 1° for 60 ± 1 minute without shaking. Remove the container from the water-bath, invert it gently ten times and transfer the contents to a glass flask. Immediately measure the *absorbance* at the maximum at about 272 nm, using the extraction solvent as blank (2.4.7).

Determine the concentration of di(2-ethylhexyl)phthalate in milligrams/100.0 ml of the extract from the calibration curve. The concentration does not exceed

— 10.0 mg per 100.0 ml for containers of nominal volume greater than 300 ml but not greater than 500 ml;

— 13.0 mg per 100.0 ml for containers of nominal volume greater than 150 ml but not greater than 300 ml;

— 14.0 mg per 100.0 ml for containers of nominal volume upto 150 ml.

Oxidisable substances. Immediately after preparation of solution A, transfer to a borosilicate-glass flask a quantity corresponding to 8 per cent of the nominal capacity of the container. At the same time, prepare a blank using an equal volume of the freshly prepared solution B in another borosilicate-glass flask. To each solution add 20.0 ml of *0.002 M potassium permanganate* and 1ml of *1 M sulphuric acid*. Allow to stand at room temperature, protected from light, for 15 minutes. To each solution add 0.1 g of *potassium iodide*. Allow to stand protected from light for 5 minutes and titrate immediately with *0.01 M sodium thiosulphate*, using 0.25 ml of *starch solution* as indicator.

The difference between the two titrations is not more than 2.0 ml.

Residue on evaporation. Evaporate to dryness 100.0 ml of solution A in a borosilicate-glass beaker, previously heated to 105°. Evaporate to dryness in the same conditions 100.0 ml of solution B. Dry to constant weight at 105°.

The difference between the weights of the residues is not more than 3 mg.

6.2.1.1.3. Sterile PVC (Polyvinyl chloride) Containers for Blood containing an Anticoagulant Solution

Unless otherwise authorised as described in the introductory part of section 6.2.1 under Containers (6.2.), the nature and composition of the material from which the containers are made complies with the requirements described under Sterile PVC (Polyvinyl chloride) containers for blood and blood components (6.2.1.1.2.).

Sterile plastic containers containing an anticoagulant solution are used for the collection, storage and administration of blood. Before filling they comply with the description and characteristics described under Sterile PVC (Polyvinyl chloride) containers for blood and blood components (6.2.1.1.2.).

After addition of the anticoagulant solution the containers comply with the tests described under Sterile Plastic Containers for Blood and Blood Components (6.2.1.1.1.) and with the following additional tests:

Light absorption. Measure the light absorption of the anticoagulant solution from the container in the range 250 nm to 350 nm using an anticoagulant solution of the same composition that has not been in contact with a plastic material as blank.

The absorbance at the maximum at about 280 nm is not more than 0.5 (2.4.7).

Extractable di(2-ethylhexyl)phthalate. Carefully remove the anticoagulant solution by means of the flexible transfer tube. Using a funnel fitted to the tube, completely fill the container with *water*, leave in contact for 1 minute, squeezing the container gently and empty completely. Repeat the rinsing.

The container then complies with the test described under Sterile PVC (Polyvinyl chloride) containers for blood and blood components (6.2.1.1.2).

Volume of anticoagulant solution. The volume does not differ by ±10 per cent from the stated volume when determined by emptying the container and collecting the anticoagulant solution in a graduated cylinder.

6.2.1.2. Plastic Containers for Non-parenteral Preparations

Tests on Containers

The container used for packaging of pharmaceutical formulations shall meet all the test requirements detailed below.

Leakage test, Collapsibility test. Comply with the tests described under Plastic Containers for Parenteral Preparations (6.2.1.1.).

The following tests are applicable to containers intended for filling oral liquids.

Clarity of aqueous extract. Select unlabelled, unmarked and non-laminated portions from suitable containers, taken at random, sufficient to yield a total area of sample required, taking into account the surface area of both sides. Cut these portions into strips, none of which has a total area of more than 20 cm^2. Wash the strips free from extraneous matter by shaking them with at least two separate portions of distilled water for about 30 seconds in each case, then draining off the water thoroughly.

Select cut and washed portions of the sample with a total surface area of 1250 cm^2, transfer to a flask, previously cleaned with *chromic acid mixture* and rinsed with several portions of *distilled water* and add 250 ml of *distilled water*. Cover the flask with a beaker and autoclave at 121° for 30 minutes. Carry out a blank determination using 250 ml of *distilled water*.

Cool and examine the extract; it is colourless and free from turbidity.

Non-volatile residue. Evaporate 100.0 ml of the extract obtained in the test for Clarity of aqueous extract to dryness and dry to constant weight at 105°.

The residue weighs not more than 12.5 mg.

Tests on Container Materials

*NOTE 6 — **Requirements for test specimens***

a) on portions of the container that are unlabelled, unprinted or non-laminated or

b) on the granules of plastic in the case of containers made by the 'form-fill-seal' process.

*NOTE 7 — **Regarding testing of extractable metals***

a) For quantitative determination of the extractable metals described hereunder, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (2.4.42) may be used. Alternatively, Atomic Absorption Spectrometry (AAS) (2.4.2) can be used provided it has the appropriate sensitivity.

b) Extractable metals are measured using special solutions as described under each plastic container.

c) Permissible limits mentioned in the paragraphs below are extractable metals in the Plastics. These have been expressed as ppm (µg of extracted metal per g of Plastics in the special solutions, after appropriate calculations).

d) The extractable elements described herein for estimation are based on their historical presence in the manufacturing processes for each plastic. Additional elements, if used by any manufacturer, should be suitably estimated.

6.2.1.2.1. Polyethylene Terephthalate (PET) Containers

Polyesters are polymers containing ester linkages generated due to the condensation of di-acids and di-ols. A prominent example in the class of polyesters is Polyethylene terephthalate (PET).

PET is a polymer which comprises at least 85 per cent units of terephthalic acid or dimethyl terephthalate condensed with ethylene glycol. Co-monomers such as isophthalic acid, dimethyl isophthalate or diethylene glycol may also be used in the PET polymerisation.

Production of PET

The polymerisation of PET is catalysed with catalyst (usually certain metal oxides), at temperatures greater than 280° and high vacuum. The manufacturing process of the PET pellets (resin) shall ensure that the residual acetaldehyde content is not greater than 10 ppm.

The resin is then given shape into bottles or any other shape through a conversion process involving injection moulding and blow moulding.

The resin and its conversion into PET containers may involve the use of colourants conforming to Indian Standards IS-9833 and/or additives conforming to IS-12252.

The manufacturers of containers should receive the certificate of analysis for each lot of resins procured from to confirm the quality of resins that the grade of resins used is as agreed and meets the relevant specification. The certificate of analysis from the resin manufacturer shall provide the necessary assurance about the quality of resin.

Quality assessment of PET Containers

The container used for packaging of pharmaceutical formulations shall meet all the test requirements detailed below.

A. IDENTIFICATION OF CONTAINER MATERIAL

Test 1 – By FTIR Spectrophotometry

Dissolve 50mg of the PET container specimen under examination in 2 ml of solvent blend of *phenol* and *tetrachloroethane* (60:40 *w/w)*with heating followed by centrifugingor *1,1,1,3,3,3-hexafluoropropan-2-ol* or other appropriate solvent systems. Apply several drops of this solution on a glass plate. Keep this plate on a water-bath in a fume cupboard to produce a thin film of about 15 mm by 15 mm. Allow the solvent to evaporate completely. Remove the film using a stream of water and a scraper. Dry the film in an oven (typically at 100-105° for about 1 hour).

Examine the film by infrared absorption spectrophotometry (2.4.6). The spectrum should show absorption maxima substantially at about 3053 cm^{-1}, 1955 cm^{-1}, 1725 cm^{-1}, 1613 cm^{-1}, 1455 cm^{-1}, 1410 cm^{-1}, 1265 cm^{-1}, 1020 cm^{-1}, 973 cm^{-1}, 875 cm^{-1}, and 730 cm^{-1}.

NOTE—Substantial, as opposed to exact, allows for minor spectral differences arising from the natural compositional and/or physical variation and/or instrumental capabilities.

Test 2 – By UV Spectrophotometry

Reflux 100.0 mg of the PET container under examination with 25.0 ml of a 20 per cent *w/v* solution of *potassium hydroxide* in a 50 per cent v/v solution of *ethanol* for 30 minutes in a round bottom flask. Allow to cool and dilute to 100.0 ml with *water*. Filter if necessary. Dilute 1.0 ml of the filtrate to 100.0 ml with *water*.

Examine this solution in the range 210 nm and 330 nm (2.4.7), the absorption maximum should be at about 240 nm.

Test 3 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from room temperature to 290° at a heating rate between10 and 20° per minute and hold it for 1 minute. Cool the specimento room temperature at the highest cooling rate possible (typically, lower than a rate of 50° per minute) and hold it for 1 minute. Reheat it to 290° at the same rate as adopted in the first heating.This reheats thermogram to be used for comparison.

Compare the thermogram of the sample with that of the reference PET. The melting peak temperature obtained from the thermogram of the sample does not differ from that of the reference thermogram by more than 8.0°.

B. CHEMICAL TESTS

B1. Tests using special solutions

Preparation of Special Solutions for Subsequent Tests on PET

Select required quantity of unlabelled, unmarked and non-laminated portions from suitable containers, taken at random. Cut them into pieces each having an area not more than $1 cm^2$.

Solution S1 (Water Extraction)

Place 10 g of the sample in a round bottom flask. Add 200.0 ml of *water* and heat at 50° for 5 hours. Allow to cool and then decant the solution.

NOTE— Use solution S1 within 4 hours of its preparation.

Solution S2 (Ethanol Extraction)

Place 10 g of the sample in a round bottom flask. Add 100.0 ml of *ethanol (95* per cent*)*and reflux at 50° for 5 hours. Allow to cool and decant the solution.

NOTE— Use solution S2 within 4 hours of its preparation.

Solution S3 (Acid Extraction)

Place 20 g of the sample in a round bottom flask. Add 50.0 ml of *0.1 M hydrochloric acid* and heat at 50° for 5 hours. Allow to cool and decant the solution.

NOTE— Use solution S3 within 4 hours of its preparation.

Solution S4 (Alkali Extraction)

Place 20 g of the sample into a round bottom flask. Add 50.0 ml of *0.01 M sodium hydroxide* and heat at 50° for 5 hours. Allow to cool and decant.

Use solution S4 within 4 hours of its preparation.

Tests using the Special Solution S1

B1.1. Appearance

Solution S1 has to be clear (2.4.1).

B1.2. Absorbance of solution S1 (2.4.7)

- In the UV- range (220 nm to 340 nm): absorbance should be not more than 0.20
- In the visible range (400 nm to 800 nm): absorbance should be not more than 0.05

B1.3. Acidity

To 50.0 ml of solution S1 add 0.15 ml of *BRP indicator solution.* The solution turns yellow. Not more than 0.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

B1.4. Alkalinity

To 50.0 ml of solution S1, add 0.2 ml of *methyl orange solution.* The solution turns yellow.

Not more than 0.5 ml of *0.01M hydrochloric acid* is required to reach the beginning of the colour change of the indicator to orange.

B1.5. Reducing substances

To 20.0 ml of solution S1, add 2.0 ml of *0.5 M sulphuric acid* and 20.0 ml of *0.002 M potassium permanganate.* Boil for 3 minutes. Immediately cool to room temperature. Add 1.0 g of *potassium iodide*, 0.25 ml of *starch solution* as indicator and titrate with *0.01 M sodium thiosulphate.* Perform a blank titration using 20.0 ml of *water.*

The difference in volume used in the 2 titrations is not greater than 0.5 ml.

Tests using the Special Solution S2

B1.6. Appearance

Solution S2 has to be clear and colourless (2.4.1).

B1.7. Absorbance of solution S2 (2.4.7)

In the visible range (400 nm to 800 nm): absorbance should be not more than 0.05.

B1.8. Extractable Metals

Tests using the Special Solution S3

B1.8.1. Aluminium. Not more than 1 ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *aluminium (200 ppm Al)* with *0.1 M hydrochloric acid.*

Wavelength. 396.15 nm, the spectral background being taken at 396.25 nm.

Verify the absence of aluminium in the *0.1 M hydrochloric acid* used.

B1.8.2. Barium. Not more than 1 ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *barium (50 ppm Ba)* with *0.1 M hydrochloric acid.*

Wavelength. 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the *0.1 M hydrochloric acid* used.

B1.8.3. Cobalt. Not more than 1 ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *cobalt (100 ppm Co)* with *0.1 M hydrochloric acid.*

Wavelength. 228.62 nm, the spectral background being taken at 228.50 nm.

Verify the absence of cobalt in the *0.1 M hydrochloric acid* used.

B1.8.4. Manganese. Not more than 1 ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *manganese (100 ppm Mn)* with *0.1 M hydrochloric acid.*

Wavelength. 257.61 nm, the spectral background being taken at 257.50 nm.

Verify the absence of manganese in the *0.1 M hydrochloric acid* used.

B1.8.5. Titanium. Not more than 1 ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *titanium (100 ppm Ti)* with *0.1 M hydrochloric acid.*

Wavelength. 323.45 nm or 334.94 nm, the spectral background being taken at 323.35 nm.

Verify the absence of titanium in the *0.1 M hydrochloric acid* used.

B1.8.6. Zinc. Not more than 1ppm.

Reference solutions. Prepare the suitably diluted solutions from reference standard solutions of *zinc (100 ppm Zn)* with *0.1 M hydrochloric acid.*

Wavelength. 213.86 nm, the spectral background being taken at 213.75 nm.

Verify the absence of zinc in the *0.1 M hydrochloric acid* used.

Tests using the Special Solution S4

B1.8.7. Antimony. Not more than 1 ppm.

Reference solutions. Prepare suitably diluted solutions from the reference standard solutions of *antimony (100 ppm Sb)* with *0.01 M sodium hydroxide.*

Wavelength. 231.15 nm or 217.58 nm, the spectral background being taken at 231.05 nm.

B2. Related Substances (Residual Monomers/Residual Inoroganics)

B2.1. Substances soluble in dioxane

Place 2.0 g of the material to be examined in a round bottom flask. Add 20.0 ml of *dioxane* and heat under reflux for 2 hours. Filter the solution. Take 10.0 ml from filtrate and evaporate to dryness on a water-bath and then dry the residue at 100-105°

The residue weighs a maximum of 30.0 mg.

B2.2. Sulphated ash (2.3.18). Not more than 0.5 per cent determined on 1.0 g.

B2.3. Total Terephthaloyl moieties. Not more than 1 ppm

Polyethylene terephthalate extracting media.(1) 50 per cent *ethanol* (dilute 125 ml of *ethanol (95 per cent)*, with *Purified Water* to 238.0 ml, and mix), (2) *n-heptane* and (3) *water.*

For each extracting media fill a sufficient number of test containers to 90 per cent of its nominal capacity to obtain not less than 30.0 ml.

Fill a corresponding number of glass bottles with each extracting medium for use as a blank. Fit the bottles with impervious seals, such as aluminium foil, or apply closures. Incubate the test packaging system and the glass bottles at 49° for 10 days. Remove the test systems and glass bottles and store at room temperature. Do not transfer the *extracting medium* samples to alternative storage vessel.

Determine the absorbance of 50 per cent ethanol extract at the wavelength of maximum absorbance at about 244 nm (2.4.7). For the blank use corresponding extracting medium blank.

Determine the absorbance of *n-heptane* extract at the wavelength of maximum absorbance at about 240 nm (2.4.7). For the blank use corresponding extracting medium blank.

The absorbance of the 50 per cent *ethanol* and *n-heptane* extracts does not exceed 0.150, corresponding to not more than 1 ppm of total terephthaloyl moieties.

B2.4. Ethylene glycol. Not more than 1 ppm.

Periodic acid solution. Dissolve 125 mg of periodic acid in 10.0 ml of *water.*

Dilute sulphuric acid. To 50.0 ml of *water*, slowly add and with constant stirring 50.0 ml of *sulphuric acid*, allow to cool to room temperature.

NOTE—Dilution of sulphuric acid produces substantial heat and can cause the solution to boil. Perform this addition carefully sulphur dioxide gas will be evolved. Use of fume hood is recommended.

Sodium bisulphite solution. Dissolve 100 mg of *sodium bisulphite* in 10.0 ml of *water.*

Disodium chromotropate solution. Dissolve 100 mg of *disodium chromotropate* in 100.0 ml of *sulphuric acid.*

Reference solution. Dissolve quantity of ethylene glycol in the *water*, to obtain a solution containing 0.0001 per cent w/v of ethylene glycol.

Test solution. Use the water extract from Total Terephthaloyl moieties

Procedure. Transfer 1.0 ml of the reference solution, test solution and purified water extracting medium in three separate volumetric flasks. Add 0.1 ml of *periodic acid solution* to each flask swirl to mix, and allow to stand for 60 minutes. Add 1.0 ml of *sodium bisulphite solution* to each flask, and mix. Add 0.1 ml of *disodium chromotropate solution* to each flask, and mix.

(*NOTE*—All the solutions should be analysed within 1 hour after addition of *disodiumchromotropate solution*) Slowly add 6.0 ml of *sulphuric acid* to each flask, mix, and allow the solutions to cool to room temperature. Dilute each solution with dilute sulphuric acid to volume, and mix. Measure the absorbance of the resulting solutions at the maximum at about 575 nm (2.4.7), using *water extracting medium* as the blank.

The absorbance of the *Sample solution* does not exceed that of the *Standard solution*, corresponding to not more than 1 ppm of *ethylene glycol.*

6.2.1.2.2. Polyethylene (HDPE and LDPE) Containers

Polyethylene is manufactured by addition polymerization of ethylene(C_2H_4), in the gas phase. The two most common types of polyethylene are high density polyethylene (HDPE) and low density polyethylene (LDPE).

Production of Polyethylene

HDPE is manufactured at pressures as low as 5 bar and low temperatures typically below 90°, using Ziegler-Natta and activated chromium oxide (known as a Phillips catalyst). The plastic identification code of HDPE is 2.

LDPE is prepared from gaseous ethylene under very high pressures upto 2500 bar andtemperatures upto 320°. These processes yield a polymer structure with both long and short branches. The plastic identification code of LDPE is 4.

The resin is given shape into bottles or any other shape through a conversion process involving injection moulding or blow moulding.

The resin and its conversion into containers may involve the use of colourants conforming to Indian Standards IS-9833 and/or additives conforming to IS-10141.

The manufacturers of containers should receive the certificate of analysis for each lot of resins procured from to confirm the quality of resins that the grade of resins used is as agreed and meets the relevant specification. The certificate of analysis from the resin manufacturer shall provide the necessary assurance about the quality of resin.

A. Identification of Container Material

High-density polyethylene (HDPE)

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 4400 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of High-Density *Polyethylene*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample and reference spectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Place about 12 mg of sample in the test specimen pan. [*NOTE– Intimate contact between the pan and the thermocouple is essential for obtaining reproducible results*]. Determine the thermogram under nitrogen, using heating/cooling conditions specified for the polymer type and using equipment capable of performing the determinations. Thermogram are obtained for the test materials and their associated reference standards.

Conduct the entire test under nitrogen. Heat the specimen from 40° to 200° at a heating rate between 2° and 10° per minute and hold it for 1 minute. Cool the specimento 40° at the same rate adopted for heatingand hold it for 1 minute. Reheat it to 200° at the same rate.This reheats thermogram to be used for comparison.

Compare the thermogram of the specimen with that of the reference HDPE. The melting peak temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 6.0°.

Low-density polyethylene (LDPE)

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 4400 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the Low-Density Polyethylene. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Method is same as for HDPE.

Conduct the entire test under nitrogen. Heat the specimen from 40° to 200° at a heating rate between 2° and 10° per minute and hold it for 1 minute. Cool the specimento 40° at the same rate adopted for heatingand hold it for 1 minute. Reheat it to 200° at the same rate. This reheats thermogram to be used for comparison.

Compare the thermogram of the specimen with that of the reference LDPE. The melting peak temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 8.0°.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polyethylene

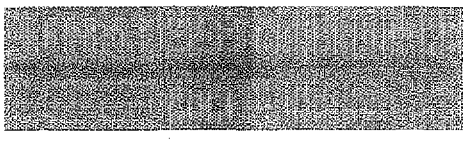
Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature, and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*.

Use Solution S1 within 4 hours of preparation.

Solution S2 (Toluene extraction)

Place 2.0 g of the test material in a 250 ml borosilicate glass flask with a round-glass neck. Add 80 ml of *toluene* and boil under a reflux condenser for 1.5 hours, stirring constantly. Allow to cool to 60° and add 120.0 ml of methanol with continued stirring. Pass the resulting solution through a sintered-glass filter. Rinse the flask and the filter with 25.0 ml of a mixture of 40 volumes of *toluene* and 60 volumes of *methanol* add the rinsing to the filtrate, and dilute to 250 ml with the same mixture of solvents to produce *Solution S2*. Prepare a blank solution.



Solution S3 (Acid extraction)

Place 5.0 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hour with constant stirring. Allow to cool and the solids to settle,decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 nm and 340 nm in Solution S1.

Absorbanceshould be not more than 0.2

B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of *BRP indicator solution*. Determine the titration volume of *0.01M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2 ml of *methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. Not more than 1.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Polymer Additives

These tests should be carried out in whole or in part as required due to the stated composition of the material.

B.3.1. Phenolic Antioxidant

Solvent mixture. Mixture of equal volumes of *acetonitrile*and *tetrahydrofuran*.

Sample solution S2A. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 5.0 ml of the solvent mixture. Prepare a blank solution from the blank solution corresponding to Solution S2.

Sample solution S2B. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the residue with 5.0 ml of *dichloromethane*. Prepare a blank solution from the blank solution corresponding to Solution S2.

Of the following reference solutions; prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). A solution containing 0.01 per cent w/v of *butylated hydroxytoluene IPRS* and 0.024 per cent w/v of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.024 per cent w/v, each of, *polymer additive 02 IPRS* and *polymer additive 03 IPRS* in the solvent mixture.

Reference solution *(c)*. A solution containing 0.024 per cent w/v, each of, *polymer additive 04 IPRS* and *polymer additive 05 IPRS* in *dichloromethane*.

Reference solution (d). A 0.01 per cent w/v solution of *butylated hydroxytoluene IPRS* in the solvent mixture.

Reference solution (e). A 0.024 per cent w/v solution of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (f). A 0.024 per cent w/v solution of *polymer additive 06 IPRS* in the solvent mixture.

Reference solution (g). A 0.024 per cent w/v solution of *polymer additive 02 IPRS* in the solvent mixture.

Reference solution (h). A 0.024 per cent w/v solution of *polymer additive 03 IPRS* in the solvent mixture.

Reference solution (i). A 0.024 per cent w/v solution of *polymer additive 04 IPRS* in *dichloromethane*.

Reference solution (j). A 0.024 per cent w/v solution of *polymer additive 05 IPRS* in *dichloromethane*.

Test A

Determine by liquid chromatography (2.4.14).

If the substance to be examined contains additive butylatedhydroxytoluene and/or additive ethylene bis[3,3-bis[3-(1,1dimethylethyl)-4-hydroxyphenyl]butanoate

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica microparticles (5µm),
- mobile phase: a mixture of 70 volumes of a *acetronitrile* and 30 volumes of *water*,
- flow rate: 2.0 ml perminute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to additive butylatedhydroxytolueneand additive ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] peaks is not less than 8.0.

Inject Sample solution S2A, corresponding blank solution, and Reference solution (d), Reference solution (e), or both. Run the chromatogram for about 30 minutes.

The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solution (d) or Reference solution (e).

NOTE— Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test B

If the substance to be examined contains one or more of the following antioxidants pentaerythritoltetrakis[3-(3,5-di tert-butyl-4-hydroxyphenyl)propionate; 2,2,23 ,6,6,63 -hexa-tert-butyl-4,4,43 -[(2,4,6-trimethyl-1,3,5-benzene-triyl)tris-methylene]triphenol; octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate; tris(2,4-di-tert-butylphenyl) phosphite; 1,3,5-tris(3,5-di-tert-butyl-4-hydroxybenzyl)-s-triazine-2,4,6(1*H*,3*H*,5*H*)-trione

Determine by liquid chromatography(2.4.14).

Chromatographic system

Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 60 volumes of *acetronitrile* 30 volumes of *tetrahydrofuran* and 10 volumes of *water,*
- flow rate: 1.5 ml per minute.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to additive pentaerythritol-tetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate and additive 2,2',23 ,6,6',63 -hexa-tert-butyl-4,4',43 -[(2,4,6-trimethyl-1,3,5-benzene-triyl)trismethylene]triphenol is not less than 2.0.

Inject Sample solution S2A, corresponding blank solution and any Reference solutions of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE— Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test C

If the substance to be examined contains additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and/or additive tris(2,4-di-tert-butylphenyl) phosphite.

Determine by liquid chromatography (2.4.14).

Chromatographic system

Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 50 volumes of *methanol*, 45 volumes of 2 *propanol* and 5.0 volumes of *water*,
- flow rate: 1.5 ml per minute.

Inject reference solution (c).The test is not valid unless the resolution between the peaks due to additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and additive tris(2,4-di-tert-butylphenyl) phosphite is not less than 2.0.

Inject Sample solution S2B, corresponding blank solution, and either Reference solutions (i) or reference solution (j) of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2B are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE — Sample solution S2B shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

B.3.2. Non-phenolic Antioxidant

Determine by thin-layer chromatography(2.4.17), using the plate coated with *silica gel GF254.*

Mobile phase A. Hexane.

Mobile phase B. dichloromethane.

Dichloromethane, acidified. To 100.0 ml of *dichloromethane* add 10.0 ml of *hydrochloric acid*, shake, allow to stand, and separate the two layers. Use the lower layer.

Sample solution S2C. Evaporate 100.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 2 ml of *dichloromethane* acidified.

Reference solution (m). A 0.6 per cent w/v solution of *polymer additive 08 IPRS* in *dichloromethane.* Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10 ml.

Reference solution (n). A 0.6 per cent w/v of *polymer additive 09 IPRS* in *dichloromethane.* Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (o). A 0.6 per cent w/v solution of *polymer additive 10 IPRS* in *dichloromethane.* Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

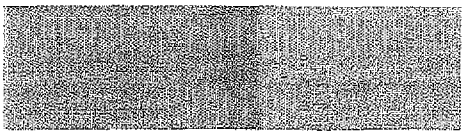
Reference solution (p). A solution containing 0.6 per cent w/v, each of, *polymer additive 10 IPRS* and *polymer additive 09 IPRS* in *dichloromethane.* Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Apply to the plate 20 µl of sample solution S2C, reference solution (p) and the reference solutions corresponding to all the phenolic and non-phenolic antioxidants expected to be present. Develop the plates over a path of 18 cm with Mobile phase A,dry in air, again develop the plate over a path of 17 cm with Mobile phase B and dry in air. Spray with alcoholic iodine solution, allow to stand for 10 to 15 minutes and examine under ultraviolet light at 254 nm.

The test is not valid unless the chromatogram obtained with reference solution (p) shows two separate spot. Any spot in the chromatogram obtained with sample solution S2C is not more intense than the spot in the same position in the chromatogram of the corresponding reference solution.

B.3.3. Amides and Stearates

Use sample solution as sample solution S2C as described in Non-phenolic Antioxidants.



Reference solution (r). A 0.2 per cent w/v solution of *stearic acid IPRS* in *dichloromethane.*

Reference solution (s). A 0.2 per cent w/v solution of *polymer additive 12 IPRS* in *dichloromethane.*

Reference solution (t). A 0.2 per cent w/v solution of *polymer additive 13 IPRS* in *dichloromethane.*

Test A

Determine by thin-layer chromatography(2.4.17), using the plate coated with silica *gel GF254*

Mobile phase. a mixture of 75 volume of *trimethylpentane* and 25 volumes of *ethanol.*

Apply to the plate 10 µl of sample solution S2C and reference solution (r). Develop the plate over a path of 10 cm with mobile phase. After development, dry the plate in air, spray with 2 per cent 2,6-dichlorophenol-indophenol sodium in dehydrated *ethanol*, heat in an oven at 120° for a few minutes to intensify the spots and examine any spot corresponding to additive stearic acid in the chromatogram obtained with sample solution S2C (R_f= about 0.5) is not more intense than the spot in the same position in the chromatogram of reference solution (r).

Test B

Determine by thin-layer chromatography (2.4.17), using the plate coated with *silica gel GF254.*

Mobile phase A. *Hexane*

Mobile phase B. A mixture of 95 volumes of *dichloromethane* and 5 volumes of *methanol.*

Apply to the plate 10 µl of sample solution S2C, reference solution (s) and reference solution (t). Develop the plateover a path of 13 cm with Mobile phase A andover a path of 10cm with Mobile phase B respectively. After each development, dry the plate in air and examine by spray with 40 per cent *phosphomolybdic acid* in *alcohol,* heat in an oven at 120° for a few minutes to intensify the spots and examineany spot corresponding to additive oleamide or erucamide in the chromatogram obtained with sample solution S2C (R_f= about 0.2) is not more intense than the spot in the same position in the chromatogram of reference solution (s) and reference solution (t).

B.4. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the total organic carbonanalysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the total organic carbon limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank total organic carbon concentrations is not more than 5 mg per litre.

B.5. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A)

Aluminium. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Arsenic, cadmium, lead, mercury, cobalt, and nickel. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g.

Chromium. Solution S3 contains not more than 0.02 mg per litre (ppm), corresponding to 0.05 µg per g.

Titanium. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Vanadium. Solution S3 contains not more than 0.04 mg per litre (ppm), corresponding to 0.1 µg per g.

Zinc. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Zirconium. Solution S3 contains not more than 0.04 mg per litre (ppm), corresponding to 0.1 µg per g.

6.2.1.2.3. Polyvinyl Chloride(PVC, Non-Plasticized) Containers

Polyvinyl Chloride is a polymer whose basic building blocks are Vinyl Chloride monomers. It is a Thermoplastic polymer.

Production of PVC

The manufacturing processes for PVC production include Suspension Polymerisation, Emulsion Polymerisation or bulk polymerisation.

The product of the polymerization process is unmodified virgin PVC. Before PVC can be made into finished products, it always requires conversion into a compound by the incorporation of additives.

PVC's major benefit is its compatibility with many different kinds of additives, making it a highly versatile polymer.

The resin and its conversion into containers may involve the use of colourants conforming to Indian Standards IS-9833 and/or additives conforming to IS-10148.

The manufacturers of containers should receive the certificate of analysis for each lot of resins procured from to confirm the

quality of resins that the grade of resins used is as agreed and meets the relevant specification. The certificate of analysis from the resin manufacturer shall provide the necessary assurance about the quality of resin.

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the *polyvinyl chloride, non-plasticized IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample and reference spectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from -20° to 120° at a heating rate between 2° and 10° per minutes. For protecting the instrument, rapidly cool the specimento room temperature.This thermogram is to be used for comparison.

Compare the thermogram of the specimen with that of the reference PVC, Non-plasticized. The glass transition temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 8.0°.

NOTE—The results of the DSC analysis are strongly dependent on the amount of plasticizer in the test article.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polyvinyl Chloride

Solution S1 (Water extraction)

Place 25 g of the test material into a borosilicate glass flask. Add 500 ml of purified water, cover the flask's neck with aluminium foil or a borosilicate beaker, and heat in an autoclave at 121 ± 2° for 20 minutes. Allow the solution to cool and the solids to settle, decant the solution into a 500-ml volumetric flask, and dilute with purified water to volume; the diluted solution is *Solution S1*.

Solution S2 (Tetrahydrofuran extraction)

Dissolve 5.0 g of the test material in 80.0 ml of *tetrahydrofuran*and dilute to a volume of 100.0 ml with the same solvent. Filter if necessary; the solution may remain opaque. Slowly and drop wise add 70 ml *ethanol* to 20.0 ml of this solution. Cool the mixture in ice for 1 hours. Filter or centrifuge the mixture, collecting residue A. Wash residue A with *ethanol*. Collect the washings and add them to the solution remaining after filtration or centrifugation. Transfer the solution to a 100-ml volumetric flask and dilute to volume with *ethanol*. This process produces *Solution S2*. Prepare a blank solution.

Solution S3 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hours with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Evaporate 100.0 ml of Solution S1 to dryness. Dissolve the residue in 5.0 ml of hexane. If necessary, pass through a filter that has been previously rinsed with hexane. Determine the spectrum between 250 nm and 330 nm in the dissolved residue.

Polyvinyl chloride, non-plasticized contains 1-phenyleicosane-1, 3-dione for dry dosage forms for oral administration

Dilute 1.0 volume of Solution S2 to 10 volumes with ethanol prior to measurement. In all other situations, analyse Solution S2 with no further preparation. Determine the spectrum between 250 and 330 nm in the dissolved residue

Absorbanceshould be not more than 0.25 for containers for non-injectable aqueous solutions.

Absorbanceshould be not more than 0.30 for containers for dry dosage forms for oral administration

B.2. Additives and Stabilizers for Polymers

The supplier of the material must be able to provide sufficient compositional information to establish whether the material meets the specifications for additives and stabilizers.

B.2.1. Tin in Tin-Stabilized Materials

Reference solution U. A 0.081 per cent w/v solution of *polymer additive 18 IPRS* in *tetrahydrofuran*. Dilute 20.0 ml to 100.0 ml with *ethanol*.

Test solution. In a test tube, add 0.1 ml of Solution S2, Add 0.05 ml of *1M hydrochloric acid*, 0.5 ml of *potassium iodide solution*, and 5.0 ml of *ethanol*. Mix thoroughly and wait for 5 minutes. Add 9 ml of *water* and 0.1 ml of a 5 g per litre solution of *sodium sulphite* and mix thoroughly.

Add 1.5 ml of *dithizone solution* freshly diluted 100-fold with *dichloromethane*, shake for 15 seconds and allow to stand for 2 minutes.

Standard solution. Use 0.1 ml of Reference solution U through the same procedure as the 0.1 ml of Solution S2.

Compare the violet colour in the lower layer of the Sample solution to the violet colour in the lower layer of the Standard solution. The colour in the Sample solution should not be as intense as the colour in the Standard solution.

Not more than 0.25 per cent w/v.

B.2.2. Tin in Non Tin-Stabilized Materials

Test solution. In a test tube, add 0.1 ml of Solution S2, Add 0.05 ml of *1Mhydrochloric acid*, 0.5 ml of *potassium iodide solution*, and 5.0 ml of *ethanol*. Mix thoroughly and wait for 5 minutes. Add 9 ml of water and 0.1 ml of a 5 g per litre solution of *sodium sulphide* and mix thoroughly.

If the solution is not colourless, add the sodium sulphate in 0.05 ml fractions

Add 1.5 ml of dithizone solution freshly diluted 100-fold with *dichloromethane*, shake for 15 seconds and allow to stand for 2 minutes.

Standard solution. Use 0.05 ml of reference solution U as per the same procedure as the 0.1 ml of Solution S2.

Compare the violet colour in the lower layer of the Sample solution to the violet colour in the lower layer of the Standard solution. The colour in the Sample solution should not be as intense as the colour in the Standard solution.

Not more than 25 µg per g (ppm).

B.3. Related Substances (Residual Monomers / Residual Solvents)

B.3.1. Vinyl Chloride

Determine by gas chromatography (2.4.13).

Test solution. Place 1.0 g of the test material in a 50.0 ml vial, and add 10.0 ml of the internal standard solution. Close the vial, and secure with a stopper. Shake by avoiding contact between the stopper and the liquid. Place the vial in a water bath at 60±1° for 2.0 hours.

Internal standard solution. Inject 10 µl of *ethyl ether* into 20.0 ml of *N,N-dimethylacetamide* by immersing the tip of the needle in the solvent using a microsyringe. Immediately before use, dilute the solution with *N,N-dimethylacetamide* to 1000 times its volume.

Vinyl chloride primary solution.

NOTE—Prepare under a ventilated hood.

Place 50.0 ml of *N,N-dimethylacetamide* in a 50-ml vial, stopper the vial, secure the stopper, and weigh to the nearest 0.1 mg. Fill a 50-ml polyethylene or polypropylene syringe with gaseous vinyl chloride, allow the gas to remain in contact with the syringe for about 3 minutes, empty the syringe, and fill again with 50.0 ml of gaseous vinyl chloride. Fit a hypodermic needle to the syringe, and reduce the volume of gas in the syringe from 50 to 25.0 ml. Inject the remaining 25.0 ml of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 hours. Store the primary solution in a refrigerator.

Vinyl chloride standard solution.

To one volume of the Vinyl chloride primary solution add three volumes of *N,N-dimethylacetamide.*

Reference solutions. Place 10.0 ml of the Internal standard solution in each of six 50.0 ml vials. Close the vials, and secure the stoppers. Inject 1, 2, 3, 5, and 10 µl, respectively, of the Vinyl chloride standard solution into five of the vials. The six solutions thus obtained contain, respectively, 0, 0.3, 0.6, 0.9, 1.5, and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water bath at 60 ± 1° for 2 hours.

Chromatographic system

- a stainless steel column 3 m x 3.0 mm, packed with with silanized diatomaceous earth for gas chromatography impregnated with 5per cent m/m of dimethylstearylamide and 5per cent m/m of *polyethylene glycol 400*,
- temperature:
  column. 45°,
  inlet port. 100°,
  detector at 150°;
- flow rate: 30 ml per minute, using nitrogen as carrier gas,

Inject 1ml of the head space of each vial containing the test solution and the Reference solutions.

Calculate the amount of vinyl chloride in the test solution by comparing the test result of the Sample solution with the test results of the reference solutions. Calculate the amount of vinyl chloride in the test material by dividing the amount of vinyl chloride in the test solution by 1.0 g, producing a result in µg per g or ppm.

Not more than 1 ppm. Note that vinyl chloride is not an additive but is monitored as a residual monomer.

B.3.1. Chlorine

50.0 mg of the test material is processed using Oxygen Flask method (2.3.34). Absorb the combustion products with 20.0 ml of *1M sodium hydroxide*. To this, add 2.5 ml of *nitric acid*, 10.0 ml of *0.1 M silver nitrate* solution, 5.0 ml of *ferric ammonium sulphate solution*, and 1ml of *dibutyl phthalate*. Titrate with *0.005Mammonium thiocyanate* solution until a reddish-yellow colour is obtained. Carry out a blank titration.

Calculate the titration volume by subtracting the volume of titrant used in the blank from the volume of titrant used in the Preparation. Each ml of *0.005 M ammonium thiocyanate* is equal to 6.25 mg of polyvinyl chloride. The chlorine content, in weight per cent, is calculated as follows:

$$\text{Chlorine content (weight per cent)} = \frac{\text{Titrant volume (ml)} \times 6.25 \text{ mg per ml}}{\text{Weight of sample (mg)}} \times 100 \text{ per cent}$$

Not less than 80 per cent by weight, expressed as polyvinyl chloride.

B.4. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the total organic carbonanalysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the total organic carbon limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank total organic carbon concentrations is not more than 5 mg per litre.

B.5. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A).

Aluminium, arsenic, lead, cadmium, mercury, cobalt, nickel, vanadium, and zinc. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01mg per litre (ppm), corresponding to less than 0.025 µg per g.

Additional acceptance criteria for materials used as containers for non-injectable aqueous solutions.

Barium. Solution S3 containsnot more than 0.10 mg per litre (ppm), corresponding to 2 µg per g.

Cadmium. Solution S3 contains not more than 0.03 mg per litre (ppm), corresponding to 0.6 µg per g.

Zinc. Solution S3 contains not more than 5 mg per litre (ppm), corresponding to 100 µg per g.

6.2.1.2.4. Polyvinyl Chloride (PVC, Plasticized) Containers

Polyvinyl Chloride is a polymer whose basic building blocks are Vinyl Chloride monomers. It is a Thermoplastic polymer.

Production of PVC

PVC is produced by Suspension Polymerisation or Emulsion Polymerisation or Bulk Polymerisation.

This virgin PVC is compounded with additives before conversion into finished products.

PVC can be plasticised using various plasticisers to make it flexible for use in medical products.

The resin and its conversion into containers may involve the use of colourants conforming to Indian Standards IS-9833 and/or additives conforming to IS-10148.

The manufacturers of containers should receive the certificate of analysis for each lot of resins procured from to confirm the quality of resins that the grade of resins used is as agreed and meets the relevant specification. The certificate of analysis from the resin manufacturer shall provide the necessary assurance about the quality of resin.

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 600 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the *polyvinyl chloride, plasticized IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from -20° to 120° at a heating rate between 2° and 10° per minutes. For protecting the instrument, rapidly cool the specimento room temperature. This thermogram is to be used for comparison.

Compare the thermogram of the specimen with that of the reference PVC, Plasticized. The glass transition temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 8.0°.

NOTE—The results of the DSC analysis are strongly dependent on the amount of plasticizer in the test article.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polyvinyl Chloride

Solution S1 (Water extraction)

Place 25 g of the test material into a borosilicate glass flask. Add 500 ml of purified water, cover the flask's neck with aluminium foil or a borosilicate beaker, and heat in an autoclave at 121 ± 2° for 20 minutes. Allow the solution to cool and the solids to settle, decant the solution into a 500-ml volumetric flask, and dilute with purified water to volume; the diluted solution is *Solution S1*.

Solution S2 (Tetrahydrofuran extraction)

Dissolve 5.0 g of the test material in 80 ml of *tetrahydrofuran* and dilute to a volume of 100.0 ml with the same solvent. Filter

if necessary; the solution may remain opaque. Slowly and drop wise add 70 ml *ethanol* to 20.0 ml of this solution. Cool the mixture in ice for 1 hour. Filter or centrifuge the mixture, collecting residue A. Wash residue A with ethanol. Collect the washings and add them to the solution remaining after filtration or centrifugation. Transfer the solution to a 100-ml volumetric flask and dilute to volume with ethanol. This process produces *Solution S2*. Prepare a blank solution.

Solution S3 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hour with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Evaporate 100.0 ml of Solution S1 to dryness. Dissolve the resulting residue in 5.0 ml of hexane to produce the hexane sample. Pass the hexane sample, if necessary, through a filter previously rinsed with hexane. Determine the spectrum between 250 and 310 nm in the hexane sample.

Absorbanceshould not bemore than 0.25.

B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of *BRP indicator solution*. Determine the titration volume of *0.01 M sodium hydroxide* required to change the colour of the indicator to blue. To 100.0 ml of Solution S1 add 0.2 ml of *Methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. Not more than 1.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Polymer Additives

Determine by thin-layer chromatography (2.4.17), using the plate coated with silica gel *GF254 (1 mm thick)*.

Additives are di(2-ethylhexyl) phthalate, N,N_3-diacylethylenediamines, epoxidized soya oil, and epoxidized linseed oil. Vinyl chloride monomer (VCM) is also monitored, although it is a residual monomer and not an additive.

Mobile Phase. Toluene.

Solution A1. Add 2.0 g of the test material to 200.0 ml of peroxide-free ether and heat under a reflux condenser for 8 hours. Separate the resulting residue B and extraction solution A by filtration. Evaporate extraction solution A to dryness under reduced pressure in a water bath at 30°, producing residue C. Dissolve residue C in 10.0 ml of toluene.

Precipitate B2. Dissolve residue B in 60 ml of *ethylene chloride* heating on a water bath under a reflux condenser, producing solution D. Filter the resulting solution D. Add the filtered solution D drop wise and with vigorous shaking to 600 ml of heptanes heated almost to boiling. Separate by hot filtration the coagulum B1 and the organic solution E. Allow solution E to cool; separate the precipitate B2 that forms upon cooling, and pass through a tared sintered-glass filter (pore size of 16–40 µm).

Reference solution (u). A 0.01 per cent w/v solution of *polymer additive 14 IPRS* in *toluene.*

Reference solution (v). A 0.01 per cent w/v solution of *polymer additive 15 IPRS* in *toluene.*

Reference solution (w). A 0.01 per cent w/v solution of *polymer additive 16 IPRS* in *toluene.*

Additive di(2-ethylhexyl) phthalate

Apply to the plate 0.5 ml of solution A to the plate as 30 mm x 30 mm. Apply 5 µl reference solution (u). Develop the plates over a path of 15 cm with toluene. After development, dry the plate in air and examine under UV light at 254 nm. Locate the zone corresponding to additive di(2-ethylhexyl) phthalate, *polymer additive 14 IPRS* (R_f about 0.4). Remove the area of silica gel corresponding to this zone, mix with 40 ml of *ethyl ether*, and shake for 1 minute. Filter, rinse filter with two quantities each of 10.0 ml of ethyl ether, add the rinsing to the filtrate, and evaporate to dryness. The residue weighs not more than 40 mg.

Residue is not more than 40 mg

Additives epoxidized soya oil and epoxidized linseed oil.

Apply to the plate 0.5 ml of solution A to the plate as 30 mm x 30 mm. Apply 5 µl reference solution (v) and reference solution (w). Develop the plates over a path of 15 cm with *toluene.* After development, dry the plate in air and expose to iodine vapor for 5 minutes. locate the zone corresponding to additive epoxidized soya oil, *polymer additive 15 IPRS* and epoxidized linseed oil, *polymer additive 16 IPRS* (R_f 0.0). Remove the area of silica gel corresponding to this band. Similarly, remove a corresponding area of silica gel as a blank reference. Separately mix both samples with separate 40 ml portions of *methanol*, shaking for 15 minutes. Filter, rinse the filter with two quantities of 10.0 ml of *methanol*, add the rinsing to the filtrate, and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

Epoxidized soya oil. The difference between the masses of both residues is not more than 10 mg.

Epoxidized linseed oil. The difference between the masses of both residues is not more than 10 mg.

B.4. Total Organic Carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the Total Organic Carbon analysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the total organic carbon limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank Total Organic Carbon concentrations is not more than 5 mg per litre.

B.5. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A).

Arsenic, cadmium, lead, mercury, cobalt, nickel, and vanadium. Report the measured value in *Solution S3* at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g.

Barium. Solution S3 contains not more than 0.25 mg per litre (ppm), corresponding to 5 µg per g.

Calcium. Solution S3 contains not more than 35 mg per litre (ppm), corresponding to 0.07 weight per cent.

Tin. Solution S3 contains not more than 1 mg per litre (ppm), corresponding to 20 µg per g.

Zinc. Solution S3 contains not more than 100 mg per litre (ppm), corresponding to 0.2 weight per cent.

B.6. Related Substances (Residual Monomers/Residual Solvents)

B.6.1. Vinyl Chloride

Internal standard solution.Using a microsyringe, Inject 10 µL of *ethyl ether* into 20.0 ml of *N,N-dimethylacetamide* by immersing the tip of the needle in the solvent. Immediately before use, dilute 1.0 volume of the solution to 1000 volumes with *N,N-dimethylacetamide*.

Test solution. Place 1.0 g of the test material in a 50.0 ml vial; and add 10.0 ml of the internal standard solution. Close the vial, and secure with a stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water bath at 60 ± 1° for 2 hours.

Vinyl chloride primary solution:

NOTE—Prepare under a ventilated hood.

Place 50.0 ml of *N,N-dimethylacetamide* in a 50-ml vial, stopper the vial, secure the stopper, and weigh to the nearest 0.1 mg. Fill a 50-ml polyethylene or polypropylene syringe with gaseous vinyl chloride, allow the gas to remain in contact with the syringe for about 3 minutes, empty the syringe, and fill again with 50.0 ml of gaseous vinyl chloride. Fit a hypodermic needle to the syringe, and reduce the volume of gas in the syringe from 50 to 25.0 ml. Inject the remaining 25.0 ml of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 hours. Store the primary solution in a refrigerator.

Vinyl chloride standard solution. To 1 volume of the *vinyl chloride primary solution*, add 3 volumes of *N,N-dimethylacetamide*.

Reference solutions . Place 10.0 ml of the Internal standard solution in each of six 50.0 ml vials. Close the vials, and secure the stoppers. Inject 1, 2, 3, 5, and 10 µl, respectively, of the Vinyl chloride standard solution into five of the vials. The six solutions thus obtained contain, respectively, 0, 0.3, 0.6, 0.9, 1.5, and 3 µg of *vinyl chloride*. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water bath at 60 ±1° for 2 hours.

Chromatographic system

- a stainless steel column 3.0 m x 3.0 mm, packed with silanized diatomaceous earth for gas chromatography impregnated with 5 per cent m/m of dimethylstearylamide and 5 per cent m/m of *polyethylene glycol 400*,
- temperature:
  column. 45°,
  inlet port. 100°,
  detector at 150°,
- flow rate: 30 ml per minute, using nitrogen as carrier gas.

Inject 1 ml of the head space of each vial containing the test solution and the reference solutions.

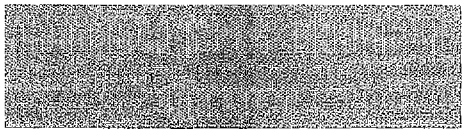
Calculate the amount of vinyl chloride in the test solution by comparing the test result of the Sample solution with the test results of the Reference solutions. Calculate the amount of vinyl chloride in the test material by dividing the amount of vinyl chloride in the test solution by 1.0 g, producing a result in µg per g or ppm.

Not more than 1ppm.

Note that vinyl chloride is not an additive but is monitored as a residual monomer.

6.2.1.2.5. Polypropylene (PP) Containers

Polypropylene is manufactured by addition polymerisation of Propylene (C_3H_6), a gaseous hydrocarbon. PP is classified as thermoplastic polymer.



Production of Polypropylene

Production of polypropylene takes place where propylene monomer is subjected to heat and pressure in the presence of a catalyst system (preferably Zeiglar Natta Catalyst). Polymerisation is achieved at relatively low temperature of around 70° and pressures of around 30 bar and the product yielded is translucent.

The resin and its conversion into containers may involve the use of colourants conforming to Indian Standards IS-9833 and/or additives conforming to IS-10909.

The manufacturers of containers should receive the certificate of analysis for each lot of resins procured from to confirm the quality of resins that the grade of resins used is as agreed and meets the relevant specification. The certificate of analysis from the resin manufacturer shall provide the necessary assurance about the quality of resin.

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the homopolymer *polypropylene IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from room temperatureto 30° above melting peak temperature (T_1) at a heating rate between 2° and 10° /minand hold it for 10 minutes. Cool the specimen at the same rateto 50° below the peak crystallization temperature and hold it for 1 minute. Reheat it to T_1 at the same rate. This reheats thermogram to be used for comparison.

Compare the thermogram of the specimen with that of the reference PP. The melting peak temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 12.0°.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polypropylene

Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature, and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*.

NOTE—Use Solution S1 within 4 hours of preparation.

Solution S2 (Toluene extraction)

Place 2.0 g of the test material in a 250-ml borosilicate glass flask with a round-glass neck. Add 80 ml of toluene and boil under a reflux condenser for 1.5 hours, stirring constantly. Allow to cool to 60° and add 120.0 ml of methanol with continued stirring. Pass the resulting solution through a sintered-glass filter. Rinse the flask and the filter with 25.0 ml of a mixture of 40 volumes of toluene and 60 volumes of methanol add the rinsing to the filtrate, and dilute to 250 ml with the same mixture of solvents to produce *Solution S2*. Prepare a blank solution.

Solution S3 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hours with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 and 340 nm in Solution S1.

Absorbanceshould be not more than 0.2.

B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of BRP indicator solution. Determine the titration volume of *0.01 M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2ml of *Methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

Not more than1.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Polymer Additives

These tests should be carried out in whole or in part as required due to the stated composition of the material.

B.3.1. Phenolic Antioxidant

Solvent mixture. Equal volumes of *acetonitrile* and *tetrahydrofuran*.

Sample solution S2A. Evaporate 50 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 5.0 ml of the Solvent mixture. Prepare a blank solution from the blank solution corresponding to Solution S2.

Sample solution S2B. Evaporate 50 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the residue with 5.0 ml of *dichloromethane*. Prepare a blank solution from the blank solution corresponding to Solution S2.

Reference solutions of the following reference solutions, prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). A solution containing 0.01 per cent w/v of *butylated hydroxytoluene IPRS* and 0.024 per cent w/v of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.024 per cent w/v, each of, *polymer additive 02 IPRS* and *polymer additive 03 IPRS* in the solvent mixture.

Reference solution *(c)*. A solution containing 0.024 per cent w/v, each of, *polymer additive 04 IPRS* and *polymer additive 05 IPRS* in *dichloromethane*.

Reference solution (d). A 0.01 per cent w/v solution of *butylated hydroxytoluene IPRS* in the solvent mixture.

Reference solution (e). A 0.024 per cent w/v solution of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (f). A 0.024 per cent w/v solution of *polymer aditive 06 IPRS* in the solvent mixture.

Reference solution (g). A 0.024 per cent w/v solution of *polymer additive 02 IPRS* in the solvent mixture

Reference solution (h). A 0.024 per cent w/v solution of *polymer additive 03 IPRS* in the solvent mixture.

Reference solution (i). A 0.024 per cent w/v solution of *polymer additive 04 IPRS* in *dichloromethane*.

Reference solution (j). A 0.024 per cent w/v solution of *polymer additive 05 IPRS* in *dichloromethane*.

Test A

Determine by liquid chromatography (2.4.14).

If the substance to be examined contains additive butylated hydroxytoluene and/or additive ethylene bis[3,3-bis[3-(1,1dimethylethyl)-4-hydroxyphenyl]butanoate.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica microparticles (5 µm),
- mobile phase: a mixture of 70 volumes of a *acetronitrile* and 30 volumes of *water*,
- flow rate: 2.0 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a).The test is not valid unless the resolution between the peaks due to additive butylated-hydroxytolueneand additive ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] peaks is not less than 8.0.

Inject Sample solution S2A, corresponding blank solution, and Reference solution (d), Reference solution (e), or both. Run the chromatogram for about 30 minutes. The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solution (d) or Reference solution (e).

NOTE—Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test B

If the substance to be examined contains one or more of the following antioxidants pentaerythrityl tetrakis[3-(3,5-di tert-butyl-4-hydroxyphenyl)propionate; 2,2,23 ,6,6,63 -hexa-tert-butyl-4,4,43 -[(2,4,6-trimethyl-1,3,5-benzene-triyl)trism-ethylene]triphenol; octadecyl 3-(3,5-di-tert-butyl-4-hydroxy-phenyl)propionate; tris(2,4-di-tert-butylphenyl) phosphate; 1,3,5-tris(3,5-di-tert-butyl-4-hydroxybenzyl)-s-triazine-2,4,6(1*H*,3*H*,5*H*)-trione

Determine by liquid chromatography (2.4.14).

Carry out the test as described in Test A with the following modifications

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica microparticles (5 µm),
- mobile phase: a mixture of 60 volumes of *acetonitrile*, 30 volumes of *tetrahydrofuran* and 10 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to additive pentaerythrityl-tetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and additive 2,2',23 ,6,6',63 -hexa-tert-butyl-4,4',43 -[(2,4,6-trimethyl-1,3,5-benzene-triyl)trismethylene]triphenol is not less than 2.0.

Inject Sample solution S2A, corresponding blank solution, and any Reference solutions of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE — Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test C

If the substance to be examined contains additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and/or additive tris(2,4-di-tert-butylphenyl) phosphite

Determine by liquid chromatography (2.4.14).

Chromatographic system

Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 50 volumes of *methanol*, 40 volumes of *2 propanol* and 5.0 volumes of *water*,
- flow rate: 1.5 ml per minute.

Inject reference solution (c).The test is not valid unless the resolution between the peaks due to additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and additive tris(2,4-di-tert-butylphenyl) phosphite peaks is not less than 2.0.

Inject Sample solution S2B, corresponding blank solution, and either Reference solutions (i) or Reference solution (j) of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2B are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE—Sample solution S2B shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

B.3.2. Non-Phenolic Antioxidant

Determine by thin-layer chromatography (2.4.17), using the plate coated with silica gel *GF254*.

Mobile phase A.Hexane.

Mobile phase B. Dichloromethane.

Dichloromethane, acidified. To 100.0 ml of *dichloromethane*, add 10.0 ml of *hydrochloric acid*, shake, allow to stand, and separate the two layers. Use the lower layer.

Sample solution S2C. Evaporate 100.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 2 ml of *dichloromethane* acidified.

Reference solution (m). A 0.6 per cent w/v solution of *polymer additive 08 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (n). A 0.6 per cent w/v solution of *polymer additive 09 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (o). A 0.6 per cent w/v solution of *polymer additive 10 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (p). A solution containing 0.6 per cent w/v, each of, *polymer additive 10 IPRS* and *polymer additive 09 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloro-methane*, acidified to 10.0 ml.

Apply to the plate 20 µl of sample solution S2C, reference solution (p) and the reference solutionscorresponding to all the phenolic and non-phenolic antioxidants expected to be present. Develop the plates over a path of 18 cm with Mobile phase A,dry in air, again develop the plate over a path of 17 cm with Mobile phase B and dry in air. Spray with alcoholic iodine solution, allow to stand for 10 to 15 minutes and examine under ultraviolet light at 254 nm. The test is not valid unless the chromatogram obtained with reference solution (p) shows two separate spot. Any spot in the chromatogram obtained with sample solution S2C is not more intense than the spot in the same position in the chromatogram of the corresponding reference solution.

B.3.3. Amides and Stearates

Use sample solution as sample solution S2C as described in Non-phenolic Antioxidants.

Reference solution (r). A 0.2 per cent w/v solution of *stearic acid IPRS* in *dichloromethane*.

Reference solution (s). A 0.2 per cent w/v solution of *polymer additive 12 IPRS* in *dichloromethane*.

Reference solution (t) A 0.2 per cent w/v solution of *polymer additive 13 IPRS* in *dichloromethane*.

Test A

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. a mixture of 75 volume of *trimethylpantane* and 25 volumes of *alcohol*.

Apply to the plate 10 µl of sample solution S2C and reference solution (r). Develop the plate over a path of 10 cm with Mobile phase. After development, dry the plate in air, spray with 2 per cent 2,6-dichlorophenol-indophenol sodium in dehydrated alcohol, heat in an oven at 120° for a few minutes to intensify the spots and examineany spot corresponding to additive stearic acid in the chromatogram obtained with sample solution S2C (R_f - about 0.5) is not more intense than the spot in the same position in the chromatogram of reference solution (r).

Test B

Determine by thin-layer chromatography(2.4.17), using the plate coated with *silica gel GF254*.

Mobile phase A. Hexane.

Mobile phase B. A mixture of 95 volumes of *dichloromethane* and 5 volumes of *methanol*.

Apply to the plate 10 µl of sample solution S2C, reference solution (s) and reference solution (t). Develop the plates over a path of 13 cm with Mobile phase A andover a path of 10 cm with Mobile phase B respectively. After each development,

dry the plate in air, spray with 40 per cent *phosphomolybdic acid* in *alcohol* heat in an oven at 120° for a few minutes to intensify the spots and examineany spot corresponding to additive oleamide or erucamide in the chromatogram obtained with sample solution S2C (R_f= about 0.2) is not more intense than the spot in the same position in the chromatogram of reference solution (s) and reference solution (t).

B.4. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the Total Organic Carbon analyses should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the total organic carbon limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank Total Organic Carbon concentrations is not more than 5 mg per litre.

B.5. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A)

Aluminium. *Solution S3* contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Arsenic, cadmium, lead, mercury, cobalt, nickel, and vanadium. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g.

Chromium. *Solution S3* contains not more than 0.02 mg per litre (ppm), corresponding to 0.05 µg per g.

Titanium. *Solution S3* contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Zinc. *Solution S3* contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

6.2.1.2.6. Containers based on Cyclic Olefins

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of *cyclic olefin polymer IPRS* or *cyclic olefin copolymer IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Given the amorphous nature of these polymers and their compositional variety, material-to-material variations in the melting peak temperature can be anticipated. Thus, it is neither recommended nor required that differential scanning calorimetry (DSC) be performed.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Cyclic Olefins

Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature, and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*.

NOTE—Use Solution S1 within 4 hours of preparation.

Solution S2 (Toluene extraction)

Place 2.0 g of the test material in a 250-ml borosilicate glass flask with a round-glass neck. Add 80 ml of toluene and boil under a reflux condenser for 1.5 hours, stirring constantly. Allow to cool to 60° and add 120.0 ml of *methanol* with continued stirring. Pass the resulting solution through a sintered-glass filter. Rinse the flask and the filter with 25.0 ml of a mixture of 40 volumes of toluene and 60 volumes of *methanol* add the rinsing to the filtrate, and dilute to 250 ml with the same mixture of solvents to produce *Solution S2*. Prepare a blank solution.

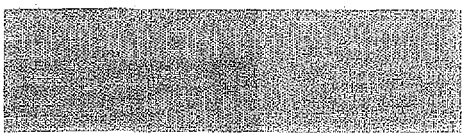
Solution S3 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hour with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 and 340 nm in Solution S1. Absorbanceshould be not more than 0.2.



B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of BRP indicator solution. Determine the titration volume of *0.01 M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2 ml of *Methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M* sodium hydroxide is required to change the colour of the indicator to blue. Not more than 1.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Polymer Additives

These tests should be carried out in whole or in part as required due to the stated composition of the material.

B.3.1. Phenolic Antioxidant

Solvent mixture. a mixture of equal volumes of *acetonitrile* and *tetrahydrofuran*.

Sample solution S2A. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 5.0ml of the Solvent mixture. Prepare a blank solution from the blank solution corresponding to Solution S2.

Sample solution S2B. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the residue with 5.0 ml of *dichloromethane*. Prepare a blank solution from the blank solution corresponding to Solution S2.

Of the following reference solutions; prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). A solution containing 0.01 per cent w/v of *butylated hydroxytoluene IPRS* and 0.024 per cent w/v of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.024 per cent w/v, each of, *polymer additive 02 IPRS* and *polymer additive 03 IPRS* in the solvent mixture.

Reference solution *(c)*. A solution containing 0.024 per cent w/v, each of, *polymer additive 04 IPRS* and *polymer additive 05 IPRS* in *dichloromethane*.

Reference solution (d). A 0.01 per cent w/v solution of *butylated hydroxytoluene IPRS* in the solvent mixture.

Reference solution (e). A 0.024 per cent w/v solution of *polymer additive 01 IPRS* in the solvent mixture.

Reference solution (f). A 0.024 per cent w/v solution of *polymer additive 06 IPRS* in the solvent mixture.

Reference solution (g). A 0.024 per cent w/v solution of *polymer additive 02 IPRS* in the solvent mixture.

Reference solution (h). A 0.024 per cent w/v solution of *polymer additive 03 IPRS* in the solvent mixture.

Reference solution (i). A 0.024 per cent w/v solution of *polymer additive 04 IPRS* in *dichloromethane*.

Reference solution (j). A 0.024 per cent w/v solution of *polymer additive 05 IPRS* in *dichloromethane*.

Test A

Determine by liquid chromatography (2.4.14).

If the substance to be examined contains additive butylatedhydroxytoluene and/or additive ethylene bis[3,3-bis[3-(1,1dimethylethyl)-4-hydroxyphenyl]butanoate

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica microparticles (5µm),
- mobile phase: a mixture of 70 volumes of *acetronitrile* and 30 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a).The test is not valid unless the resolution between the peaks due to additive butylatedhydroxytolueneand additive ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] peaks is not less than 8.0.

Inject Sample solution S2A, corresponding blank solution, and Reference solution (d), Reference solution (e), or both. Run the chromatogram for about 30 minutes. The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solution (d) or Reference solution (e).

NOTE — Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test B

If the substance to be examined contains one or more of the following antioxidants pentaerythrityltetrakis[3-(3,5-di tert-butyl-4-hydroxyphenyl)propionate; 2,2,23 ,6,6,63 -hexa-tert-butyl-4,4,43 -[(2,4,6-trimethyl-1,3,5-benzene-triyl) tris-methylene]triphenol; octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate; tris(2,4-di-tert-butylphenyl) phosphite; 1,3,5-tris(3,5-di-tert-butyl-4-hydroxybenzyl)-s-triazine-2,4,6(1*H*,3*H*,5*H*)-trione

Chromatographic system Determine by liquid chromatography (2.4.14). Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 60 volumes of *acetronitrile* 30 volumes of *tetrahydrofuran* and 10 volumes of *water*,
- flow rate: 1.5 ml per minute.

Inject reference solution (b).The test is not valid unless the resolution between the peaks due to additive pentaerythrityl-tetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and additive 2,2',23 ,6,6',63 -hexa-tert-butyl-4,4',43 -[(2,4,6-

trimethyl-1,3,5-benzene-triyl)trismethylene]triphenol peaks is not less than 2.0.

Inject Sample solution S2A, corresponding blank solution,and any Reference solutions of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2A are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE—Sample solution S2A shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

Test C

If the substance to be examined contains additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and/or additive tris(2,4-di-tert-butylphenyl) phosphite.

Chromatographic system Determine by liquid chromatography (2.4.14). Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 50 volumes of *methanol*, 40 volumes of *2 propanol* and 5.0 volumes of *water,*
- flow rate: 1.5 ml per minute.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to additive octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate and additive tris(2,4-di-tert-butylphenyl) phosphite peaks is not less than 2.0.

Inject Sample solution S2B, corresponding blank solution, and either Reference solutions (i) or Reference solution (j) of the antioxidants listed above that are stated in the composition. The peak areas of Sample solution S2B are less than the corresponding peak areas of Reference solutions of the antioxidants that are listed above and that are stated in the composition.

NOTE—Sample solution S2B shows only peaks caused by antioxidants stated in the composition and minor peaks that also correspond to the blank solution.

B.3.2. Non-phenolic Antioxidant

Determine by thin-layer chromatography(2.4.17), using the plate coated with silica *gel GF254.*

Mobile phase A. Hexane.

Mobile phase B. Dichloromethane.

Dichloromethane, acidified. To 100.0 ml of *dichloromethane* add 10.0 ml of *hydrochloric acid*, shake, allow to stand, and separate the two layers. Use the lower layer.

Sample solution S2C. Evaporate 100.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 2 ml of *dichloromethane*, acidified.

Reference solution (m). 0.6 per cent w/v solution of *polymer additive 08 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (n). A 0.6 per cent w/v solution of *polymer additive 09 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (o). A 0.6 per cent w/v solution of *polymer additive 10 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloromethane*, acidified to 10.0 ml.

Reference solution (p). A solution containing 0.6 per cent w/v, each of, *polymer additive 10 IPRS* and *polymer additive 09 IPRS* in *dichloromethane*. Dilute 2.0 ml of the solution with *dichloro-methane*, acidified to 10.0 ml.

Apply to the plate 20 µl of sample solution S2C, reference solution (p) and the reference solutions corresponding to all the phenolic and non-phenolic antioxidants expected to be present. Develop the plates over a path of 18 cm with Mobile phase A anddry in air, again develop the plate over a pan of 17 cm with Mobile phase B and dry in air. Spray with alcoholic iodine solution, allow to stand for 10 to 15 minutes and examine under ultraviolet light at 254 nm.

The test is not valid unless the chromatogram obtained with reference solution (p) shows two separate spot. Any spot in the chromatogram obtained with sample solution S2C is not more intense than the spot in the same position in the chromatogram of the corresponding reference solution.

B.3.3. Copolymer of Dimethyl Succinate and (4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-yl) ethanol

Determine by liquid chromatography (2.4.14).

Solvent mixture. 89 volume of *hexane* and 11 volumes of *methanol.*

Sample solution S2D. Evaporate 25 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the residue with 10 ml of *toluene* and 10 ml of a 10 per cent w/v solution of *tetrabutyl ammonium hydroxide* in a mixture of 35 volumes of *toluene* and 65 volumes of *anhydrous methanol*. Boil under a reflux condenser for 3 hours. Allow to cool, and filter.

Reference solution (q). A 0.06 per cent w/v solution of *polymer additive 11 IPRS* in *toluene*. Add 1.0 ml of this solution to 25 ml of the blank solution corresponding to Solution S2 and evaporate to dryness under vacuum at 45°. Prepare a blank solution from the blank solution corresponding to solution S2. Dissolve the residue with 10 ml of toluene and 10 ml of a 10 per cent w/v solution of *tetrabutyl ammonium hydroxide* in a mixture of 35 volumes of *toluene* and 65 volumes of *anhydrous methanol*. Boil under a reflux condenser for 3 hours. Allow to cool, and filter.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica microparticles (5 µm),
- mobile phase: a mixture of 89 volume of *hexane* and 11 volumes of *methanol,*
- flow rate: 2 ml per minute,

- spectrophotometer set at 227 nm,
- injection volume: 20 µl.

Inject reference solution (q). The test is not valid unless the resolution between the peaks due to diol component and the diluents peaks is not less than 7.0.

Inject Sample solution S2D corresponding blank solution and Reference solution (q). Run the chromatogram for about 30 minutes. The peak areas of diol component in Sample solution S2A are less than the corresponding peak areas of teference solution (q).

B.3.4. Amides and Stearates

Use sample solution as sample solution S2C as described in Non-phenolic Antioxidants.

Reference solution (r). A 0.2 per cent w/v solution of *stearic acid IPRS* in *dichloromethane.*

Reference solution (s). A 0.2 per cent w/v solution of *polymer additive 12 IPRS* in *dichloromethane.*

Reference solution (t). A 0.2 per cent w/v solution of *polymer additive 13 IPRS* in *dichloromethane.*

Test A

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. a mixture of 75 volumes of *trimethylpentane* and 25 volumes of *alcohol.*

Apply to the plate 10 µl of sample solution S2C and reference solution (r). Develop the plate over a path of 10 cm with Mobile phase. After development, dry the plate in air, spray with 2 per cent 2,6-dichlorophenol-indophenol sodium in dehydrated alcohol, heat in an oven at 120° for a few minutes to intensify the spots and examineany spot corresponding to additive stearic acid in the chromatogram obtained with sample solution S2C (R_f = about 0.5) is not more intense than the spot in the same position in the chromatogram of reference solution (r).

Test B

Determine by thin-layer chromatography (2.4.17), using the plate coated with silica *gel GF254.*

Mobile phase A. Hexane.

Mobile phase B. A mixture of 95 volumes of *dichloromethane* and 5 volumes of *methanol.*

Apply to the plate 10 µl of sample solution S2C, reference solution (s) and reference solution (t). Develop the plates over a path of 13 cm with Mobile phase A andover a path of 10 cm with Mobile phase B respectively. After each development, dry the plate in air, spray with 40 per cent *phosphomolybdic acid* in *alcohol,* heat in an oven at 120° for a few minutes to intensify the spots and examine any spot corresponding to additive oleamide or erucamide in the chromatogram obtained with sample solution S2C (R_f = about 0.2) is not more intense than the spot in the same position in the chromatogram of reference solution (s) and reference solution (t).

B.4. Total Organic Carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the Total Organic Carbon analyses should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the TOC limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank total organic carbon concentrations is not more than 5 mg/L.

B.5. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A)

Aluminium. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Arsenic, cadmium, lead, mercury, cobalt, nickel, and vanadium. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g.

Titanium. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

Zinc. Solution S3 contains not more than 0.4 mg per litre (ppm), corresponding to 1 µg per g.

6.2.1.2.7. Poly(Ethylene-vinyl Acetate) (PEVA) Containers

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 4400 cm^{-1} to 650 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of *poly(ethylene-vinyl acetate) IPRS.* Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from "50° to 120° at a heating rate between 2° and 10° /minute. For protecting the instrument,rapidly cool the specimento 40° at the same rate.

Compare the thermogram of the specimen with that of the reference PEVA. The transition temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 6.0°.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Poly(ethylene-vinyl acetate)

Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature, and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*.

NOTE — Use Solution S1 within 4 hours of preparation.

Solution S2 (Toluene extraction)

Place 2.0 g of the test material in a 250-ml borosilicate glass flask with a round-glass neck. Add 80 ml of toluene and boil under a reflux condenser for 1.5 hours, stirring constantly. Allow to cool to 60° and add 120.0 ml of *methanol* with continued stirring. Pass the resulting solution through a sintered-glass filter. Rinse the flask and the filter with 25.0 ml of a mixture of 40 volumes of *toluene* and 60 volumes of *methanol* add the rinsing to the filtrate, and dilute to 250 ml with the same mixture of solvents to produce *Solution S2*. Prepare a blank solution.

Solution S3 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hours with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S3*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 and 340 nm in Solution S1.

Absorbance should be not more than 0.2.

B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of *BRP indicator solution*. Determine the titration volume of *0.01 M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2 ml of *Methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Polymer Additives

These tests should be carried out in whole or in part as required due to the stated composition of the material.

B.3.1. Phenolic Antioxidants

Solvent mixture. A mixture of equal volumes of *acetonitrile* and *tetrahydrofuran.*

Sample solution S2E. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 5.0 ml of the Solvent mixture. Prepare a blank solution from the blank solution corresponding to Solution S2.

Sample solution S2F. Evaporate 50.0 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the residue with 5.0 ml of *dichloromethane*. Prepare a blank solution from the blank solution corresponding to Solution S2.

Reference solution (k). A solution containing 0.01 per cent w/v of *butylated hydroxy toluene IPRS*, 0.016 per cent w/v of *polymer additive 02 IPRS*, 0.016 per cent w/v of *polymer additive 03 IPRS* and 0.016 per cent w/v of *polymer additive 04 IPRS* in the solvent mixture.

Reference solution(l). A solution containing 0.016 per cent w/v, each of, *polymer additive 04 IPRS* and *polymer additive 05 IPRS* in *dichloromethane.*

NOTE — Of the following reference solutions, prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Test A

Determine by liquid chromatography (2.4.14).

If the substance to be examined contains additive butylatedhydroxytoluene and/or additive ethylene bis[3,3-bis[3-(1,1dimethylethyl)-4-hydroxyphenyl]butanoate

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica microparticles (5 µm),
- mobile phase: a mixture of 70 volumes of *tetrahydrofuran,* 60 volumes of *acetronitrile*, and 10 volumes of *water,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (k).The test is not valid unless the resolution between the peaks due to polymer additive 02 and polymer additive 03 peaks is not less than 2.0 and the column efficiency is not less than 2500 theoretical plates calculated for butylated hydroxytoluene.

Inject Sample solution 12 corresponding blank solution, reference solution (k), Run the chromatogram for about 30 minutes. The peak areas of Sample solution 12 are less than the corresponding peak areas of reference solution (k).

NOTE—Sample solution S2E shows only peaks caused by antioxidants stated in the reference solution (k) and minor peaks that also correspond to the blank solution.

Test B

If the chromatogram obtained via Test A for Test solution S2E shows a peak with the same retention time as the last antioxidant eluted from Reference solution K.

Determine by liquid chromatography (2.4.14).

Chromatographic system

Carry out the test as described in Test A with the following modifications

- mobile phase: a mixture of 45 volumes *of 2 propanol* 50 volumes of *methanol* and 5.0 volumes of *water*,
- flow rate: 1.5 ml per minute.

Inject reference solution (l).The test is not valid unless the resolution between the peaks due to polymer additive 04 and polymer additive 05 peaks is not less than 2.0.

Inject Sample solution S2F corresponding blank solution, reference solution (l). The peak areas of Sample solution S2F are less than the corresponding peak areas of reference solution (l).

NOTE—Sample solution 13 shows only peaks caused by antioxidants stated in the reference solution (l) and minor peaks that also correspond to the blank solution.

B.3.2. Amides and Stearic Acid

Sample solution S2G: Evaporate 100 ml of Solution S2 to dryness under vacuum at 45°. Dissolve the resulting residue with 2.0 ml of acidified *dichloromethane*.

Reference solution (r) A 0.2 per cent w/v solution of *stearic acid IPRS* in *dichloromethane*.

Reference solution (s) A 0.08 per cent w/v solution of *polymer additive 12 IPRS* in *dichloromethane*.

Reference solution (t) A 0.08 per cent w/v solution of *polymer additive 13 IPRS* in *dichloromethane*.

Test A

Determine by thin-layer chromatography(2.4.17), using the plate coated with *silica gel GF254*.

Mobile phase. a mixture of 75 volume of *trimethylpantane* and 25 volumes of *ethanol*.

Apply to the plate 10 µl of sample solution S2G, reference solution (r). Develop the plates over a path of 10 cm with mobile phase. After development, dry the plate in air and examine by spray with 2.0 per cent w/v solution of *2, 6-dichlorophenol-indophenol sodium* in dehydrated *ethanol* and heat in an oven at 120° for a few minutes to intensify the spots. Any spot corresponding to additive stearic acid in the chromatogram obtained with sample solution S2G is not more intense than the spot in the same position in the chromatogram of the corresponding reference solution (r).

Test B

Determine by thin-layer chromatography(2.4.17), using the plate coated with *silica gel GF254*.

Mobile phase A. *Hexane*.

Mobile phase B. A mixture of 95 volumes of *dichloromethane* and 5 volumes of *methanol*.

Apply to the plate 10 µl of sample solution S2G, reference solution (s) and reference solution (t). Develop the plates over a path of 13 cm with mobile phase A and over a path of 10cm with Mobile phase B respectively. After development, dry the plate in air and examine by spray with 40 per cent *phosphomolybdic acid* in *alcohol* heat in an oven at 120° for a few minutes to intensify the spots. Any spot corresponding to additive oleamide or erucamide in the chromatogram obtained with sample solution S2G is not more intense than the spot in the same position in the chromatogram of reference solution (s) and reference solution (t).

B.4. Related Substances (Residual Monomers / Residual Solvents)

B.4.1. Vinyl Acetate

Test solution: Place 0.25 to1.0 g of the test material into a 300 ml conical flask containing a magnetic stirrer. Prepare an extraction blank starting with an otherwise empty 300 ml conical flask. Add 40 ml of *xylene* and boil under a reflux condenser with stirring for 4 hours. After heating, continue stirring, allowing the solution to cool to the point that precipitation starts. Slowly add 25.0 ml of alcoholic *potassium hydroxide*. Boil again under a reflux condenser for 3 hours with continued stirring. While stirring, allow the solution to cool, rinse the condenser with 50.0 ml of water and add 30 ml of *0.05 M sulphuric acid* to the flask. Transfer the contents of the flask to a 400-ml beaker, rinsing the flask with the following: 2.0 volumes, 50.0 ml each, of a 200 g per litre solution of *anhydrous sodium sulphate* and 3.0 volumes, 20.0 ml, each of, *water*. Add the rinsings to the flask.

Titrate the excess sulphuric acid in Test solution with *0.1 M sodium hydroxide*, determining the endpoint potentiometrically (2.4.25). Carry out a titration of the extraction blank.

Determine the amount of titrant (ml) required by subtracting the titrant volume used for the extraction blank (ml) from the titrant volume used for the extract (ml). Determine the amount of vinyl acetate by multiplying the volume of titrant required by the quantity 8.609 mg/ml. The content of vinyl acetate is calculated as:

$$\text{Content of vinyl acetate (weight per cent)} = \frac{\text{Amount of vinyl acetate (mg)}}{\text{Weight of material extracted (g)}} \times 10$$

Content of vinyl acetate

For containers. Not more than 25 per cent by weight.

For tubing. Not more than 30 per cent by weight.

B.5. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the Total Organic Carbonanalysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the TOC limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank Total Organic Carbon concentrations is not more than 5 mg per litre.

B.6. Extractable Metals

Solution S3 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A).

All metals. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g.

6.2.1.2.8. Polycarbonate (PC) Containers

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 600 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the *polycarbonate IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample and reference spectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from -20° to 150° at a heating rate between 2° and 10° per minutes. For protecting the instrument,rapidly cool the specimen to room temperature. This thermogram is to be used for comparison.

Compare the thermogram of the specimen with that of the reference Polycarbonate. The glass transition temperature obtained from the thermogram of the specimen does not differ from that of the reference thermogram by more than 8.0°.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polycarbonate

Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*.

NOTE—Use Solution S1 within 4 hours of preparation.

Solution S2 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0 ml of *0.1 M hydrochloric acid*, and boil under a reflux condenser for 1 hours with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1 M hydrochloric acid*; the diluted solution is *Solution S2*.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 and 340 nm in Solution S1

Absorbance should be not more than 0.20

B.2. Acidity or alkalinity

To 100.0 ml of Solution S1 add 0.15 ml of *BRP indicator solution*. Determine the titration volume of *0.01M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2 ml of *Methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Related Substances (Residual Monomers / Residual Solvents)

B.3.1. Residual Solvents

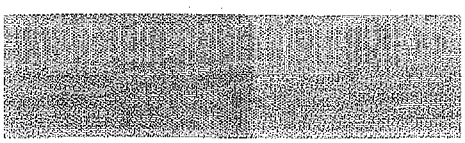
By Head Space Gas Chromatography (2.4.13).

Test solution. Weigh 1.0 g of the test material and place it in a 20-ml headspace vial. Add 10.0 ml of *N, N,-dimethylformamide*, cap the vial closed, and sonicate for 4 hours. Cool to room temperature. Prepare a sample blank in a similar fashion.

Residual solvents primary solution. Weigh 500 mg each of *dichloromethane*, *toluene*, and *ethylbenzene*and 1250 mg of *chlorobenzene* into a 50-ml volumetric flask; dissolve and adjust with *N,N,-dimethylformamide* to volume.

Residual solvents stock solution. Transfer 5.0 ml of the Residual solvents primary solution into a 100-ml volumetric flask; adjust with *N, N-dimethylformamide* to volume.

Reference solutions. Pipet 0, 2, 3, 4, 5, and 6 ml of the Residual solvents stock solution into individual 100-ml volumetric flasks, dilute with *N-N-dimethylformamide* to volume and mix



well. The six reference standards thus obtained (Reference solution blank and WS1 through WS5) contain, respectively, 0, 10, 15, 20, 25, and 30 mg per litre of *dichloromethane, toluene*, and *ethylbenzene*and 0, 37.5, 50, 62.5, and 75 mg per litre of *chlorobenzene*.

Chromatographic system

- a capillary column 30 m x 0.32 mm, packed with 100 per cent bonded and cross linked polyethylene glycol (5 µm),
- temperature:
  column: Start at 50°, hold for 20 minutes. Heat to 165° at 6° per minute, hold for 20 minutes,
- inlet port.140°,
- detector 250°,
- flame ionization detector
- flow rate: adjust to provide a constant pressure of 10 psi, using helium or nitrogen as carrier gas
- injection volume: 1 µl, split.

Head space conditions

- temperatures:
  thermostating: 115°,
  needle: 110°,
  transfer: 120°,
- thermostating: 60 minutes,
- pressurization, 0.5 minutes,
- injection, 0.1 minute,
- withdrawal, 0.2 minute,
- carrier gas pressure, 20 psi.

Conditioning. Inject the reference solution blank three times into the chromatographic system.

Inject reference solution WS3 five times into the chromatographic system. The test is not valid unless, the relative standard deviation of the peak areas in the chromatogram obtained with reference solution WS3 for is not less than 5.0 per cent. The symmetry factor for the caprolactam peak obtained for the third injection must be between 0.8 and 1.3.

Rinsing. Inject the reference solution blank once.

Calibration, front of bracket. Inject each of the five Reference solutions once. Construct a linear calibration curve of the peak areas obtained for the Reference solutions versus their analyte concentrations for each analyte. The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not less than 0.99.

Rinsing. Inject the reference solution blank once.

Sample. Inject each test solution once, including the blank. Inject not more than six test solutions.

Rinsing. Inject Reference solution blank once.

Calibration, back of bracket. Inject each of the five reference solutions once.

Calculations: Construct a linear calibration curve of the peak areas obtained for the Reference solutions versus their analyte concentrations (using the front and back of the bracket). The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not more than 0.99. Calculate the amount of each analyte in the test solution by putting the peak area obtained for the test solution into the calibration curve.

Calculate the amount of each in the test material by multiplying this result by a factor of 10 and dividing the product by the weight of the test material in g, producing a result in µg per g.

$$\text{Analyte (µg per g)} = \frac{\text{Analyte in sample solution (mg per litre)}}{\text{Weight of test material (g)}} \times 10$$

B.3.1. Bisphenol A

NOTE—Bisphenol A is monitored although it is a residual monomer and not an additive.

Test solution. Weigh 1.0 g of the test material and place it in a 250 ml round-bottom flask. Add 50.0 ml of *dichloromethane* and heat at 50° for 1 hour under a reflux condenser to dissolve the test material. Cool the solution to room temperature and slowly add 75 ml of *methanol* to the room temperature solution, with continuous stirring. Place in a refrigerator for 2 hours to cool the resulting solution. Filter the cooled solution through a sintered-glass filter. Wash the round-bottom flask and the filter twice with 15.0 ml of *methanol*. Evaporate the filtrate to dryness under vacuum at 45°. Dissolve the residue in 5.0 ml of *dichloromethane*. Add 0.5 ml of this solution and 0.5 ml of N,O-bis(trimethylsilyl)trifluoroacetamide to a 1.5-ml vial and close the vial immediately. Heat the closed vial at 40° for 2 hours and then cool to room temperature. Prepare a sample blank in a similar fashion.

Bisphenol A primary solution. Weigh 20 mg of *bisphenol A IPRS* in a 200-ml volumetric flask; dissolve and dilute with *dichloromethane* to volume.

Reference solutions. Pipet 0, 5, 10, 20, 30, and 40 ml of the BisphenolA primary solution into six 100-ml volumetric flasks. Dilute with *dichloromethane* to volume and mix well. The six reference standards thus obtained (Reference solution blank and WS1 through WS5) contain, respectively, 0, 5, 10, 20, 30, and 40 mg per litre of Bisphenol A.

Add 0.5 ml each of the reference solutions and 0.5 ml of *N,O-bis(trimethylsilyl)trifluoroacetamide* to separate 1.5-ml vials and close the vials immediately. Heat the closed vials at 40° for 2 hours and then cool to room temperature.

Chromatographic system

- a capillary column 25 m x 0.25 mm, packed with 100 per cent dimethylpolysilxane (0.25 µm),
- temperature:
  column: 250°,
  inlet port detector 300°,
- flame ionization detector,

- flow rate: adjust to provide a constant pressure of 13 psi, using helium as carrier gas
- injection volume: 2 µl, split.

Head space conditions

- temperatures:
  thermostating: 115°,
  needle: 110°,
  transfer: 120°,
- thermostating, 60 minutes,
- pressurization, 0.5 minute,
- injection, 0.1 minute,
- withdrawal, 0.2 minute,
- carrier gas pressure, 20 psi.

Conditioning. Inject the reference solution blank three times into the chromatographic system.

Inject reference solution WS3 five times into the chromatographic system. The test is not valid unless, the relative standard deviation of the peak areas in the chromatogram obtained with reference solution WS3 for is not less than 5.0 per cent.

Rinsing. Inject the reference solution blank twice.

Calibration, front of bracket: Inject each of the five reference solutions once. Construct a linear calibration curve of the peak areas obtained for the reference solutions versus their bisphenol A concentrations. The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not less than 0.98.

Rinsing. Inject the reference solution blank once.

Sample. Inject each test solution once, including the sample blank. Inject not more than six test solutions.

Rinsing. Inject the reference solution blank once

Calibration, back of bracket: Inject each of the five reference solutions once.

Calculations. Construct a linear calibration curve of the peak areas obtained for the reference solutions versus their bisphenolA concentrations (front and back of bracket). The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not less than 0.98. Calculate the amount of bisphenol A in the Sample solution by putting the peak area obtained for the Sample solution into the calibration curve. Calculate the amount of bisphenol A in the test material by multiplying this result by a factor of five and dividing the product by the weight of the test material in g, producing a result in µg per g,

$$\text{Bisphenol A (µµ per g)} = \frac{\text{Bisphenol A in test solution (mg per litre)}}{\text{Weight of test material}} \times 5$$

Not more than 100 µg per g.

B.4. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the TOC analysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the TOC limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank Total Organic Carbon concentrations is not more than 5 mg per litre.

B.5. Extractable Metals

Solution S2 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A).

Arsenic, lead, cadmium, mercury, cobalt, nickel, and vanadium. Report the measured value in Solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g. Additional acceptance criteria for certain metals are provided as follows.

6.2.1.2.9. Polyamide-6 (PA-6) Containers

A. Identification of Container Material

Test 1 – By FTIR Spectrophotometry (2.4.6)

Determine the infrared spectrum from 3800 cm^{-1} to 600 cm^{-1}. The specimen exhibits an absorption spectrum that is substantially equivalent to that of the *polyamide-6 IPRS*. Substantial, as opposed to exact, equivalence allows for minor spectral differences arising from the natural compositional and/or physical variation among polymers of this class. Substantial equivalence is achieved when all differences between the sample andreferencespectra can be explained in the context of such natural compositional and/or physical variations.

Test 2 – By Differential Scanning Calorimetry (2.4.31)

Conduct the entire test under nitrogen. Heat the specimen from room temperature to 250° at a heating rate between 10 and 20° per minute and hold it for 1 minute. Cool the specimento room temperature at the highest cooling rate possible (typically, lower than a rate of 50° per minutes) and hold it for 1 minute. Reheat it to 250° at the same rate as adopted in the first heating.This reheat thermogram to be used for comparison.

Compare the thermogram of the sample with that of the reference Polyamide-6. The melting peak temperature obtained from the thermogram of the sample does not differ from that of the reference thermogram by more than 8.0°.

B. Chemical Tests

Preparation of Special Solutions for subsequent tests on Polyamide-6

Solution S1 (Water extraction)

Place 25 g of the test material in a borosilicate glass flask with a round-glass neck. Add 500 ml of purified water, and boil under reflux conditions for 5 hours. Allow to cool to ambient temperature, and filter the extracting solution through a sintered-glass filter. Collect the filtrate in a 500-ml volumetric flask and dilute with purified water to volume; the diluted solution is *Solution S1*. Use *Solution S1* within 4 hours of preparation.

Solution S2 (Acid extraction)

Place 5 g in a borosilicate glass flask with a round-glass neck. Add 100.0ml of *0.1M hydrochloric acid*, and boil under a reflux condenser for 1 hours with constant stirring. Allow to cool and the solids to settle, decant the solution into a 100-ml volumetric flask, and dilute to volume with *0.1M hydrochloric acid*; the diluted solution is *Solution S2*.

Solution S3 (Phenol extraction)

Dissolve 1.0 g of the test material in 50.0 ml of phenol by heating at 50° for 4 hours with constant stirring. This process produces *Solution S3*. Prepare a blank solution.

Tests Using Special Solutions

B.1. Absorbance (2.4.7)

Determine the spectrum between 220 and 340 nm in Solution S1.

Absorbance should be not more than 0.25

B.2. Acidity or alkalinity

To 100 ml of Solution S1 add 0.15 ml of BRP indicator solution. Determine the titration volume of *0.01 M sodium hydroxide* required to change the colour of the indicator to blue. To a separate, 100.0 ml portion of Solution S, add 0.2 ml of *methyl orange solution*. Determine the titration volume of *0.01 M hydrochloric acid* required to reach the beginning of the colour change of the indicator from yellow to orange.

Not more than 1.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

Not more than 4.0 ml of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

B.3. Free Base Functions

Perchloric acid in 70 per cent phenol. Dissolve approximately 0.72 g (target 0.710–0.7250 g) of *perchloric acid* in 50.0 ml of phenol.

Titrate 50.0 ml of solution S3 with Perchloric acid in 70 per cent *phenol*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration using phenol as extraction blank.

The difference between the titration volumes, extract versus extraction blank, is not more than 0.4 ml.

B.4. Related Substances (Residual Monomers/Residual Solvents)

B.4.1. Caprolactam

Determine by gas chromatography (2.4.13).

Test Solution. Dissolve 1.0 g of the test substance in sufficient quantity of *formic acid*. Dilute to 10.0 ml with same solvent.

Caprolactam primary solution. Dissolve 125 mg of *Caprolactam* reference in *formic acid* and dilute to 50.0 ml with same solvent.

Reference solutions. Pipet 0, 2, 4, 6, 8, and 10.0 ml of the Caprolactam primary solution into six 20-ml volumetric flasks. Dilute with anhydrous formic acid to volume. The six reference standards thus obtained (blank and WS1 through WS5) contain, respectively, 0, 250, 500, 750, 1000, and 1250 mg per litre of caprolactam.

Chromatographic system

- a capillary column 2 m x 4 mm, packed with silanized diatomaceous earth for gas chromatography impregnated with 10 per cent m/m of polyethylene glycol 20000,
- temperature:
  column. 170°,
  inlet port detector 250°,
- flame ionization detector
- flow rate 25 ml per minute, using helium or nitrogen as carrier gas,
- injection volume: 1 µl.

Inject 1 µl of the reference solution blank three times into the chromatographic system.

Inject reference solution WS4 five times into the chromatographic system. The test is not valid unless, the relative standard deviation of the peak areas in the chromatogram obtained with reference solution WS4 for is not less than 5 per cent. The symmetry factor for the caprolactam peak obtained for the third injection must be between 0.8 and 1.3.

Rinsing. Inject 1 µl of the reference solution blank once.

Calibration, front of bracket. Inject each of the five reference solutions once. Construct a linear calibration curve of the peak areas obtained for the Reference solutions versus their caprolactam concentrations. The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not less than 0.98.

Rinsing. Inject 1 µl of the reference solution blank once.

Sample. Inject 1 µl each test solution once. Inject not more than six test solutions.

Rinsing. Inject 1 µl of the reference solution blank once.

Calibration, back of bracket. Inject 1 µl each of the five reference solutions once.

Construct a linear calibration curve of the peak areas obtained for the reference solutions versus their caprolactam concentrations (both front and back of bracket). The correlation coefficient (r^2) obtained for the best-fit linear regression line must be not less than 0.98. Calculate the amount of caprolactam

in the test solution by putting the peak area obtained for the test solution into the calibration curve. Calculate the amount of caprolactam in the test material by multiplying this result by a factor of 10 and dividing the product by the weight of the test material in g, producing a result in weight per cent.

Not more than1 per cent.

B.5. Total organic carbon

The total organic content of Solution S1 is measured according to the general methodologies outlined in Total Organic Carbon (2.4.30).

The method used to perform the Total Organic Carbon analysis should have a limit of detection of 0.2 mg per litre (ppm) and should have a demonstrated linear dynamic range from 0.2 to 20 mg per litre (which encompasses the Total Organic Carbon limit). A linear range with a higher upper concentration can be used if linearity is established. If sample extracts exceed this upper linear range, they must be diluted appropriately for analysis.

The difference between the sample and blank Total Organic Carbon concentrations is not more than 5 mg per litre.

B.6. Extractable Metals

Solution S2 is used for acid extractable metals.

Procedure for extract analysis

Instrumentation and methods are those specified in Elemental Impurities (2.3.13A)

Arsenic, lead, cadmium, mercury, cobalt, nickel, and vanadium. Report the measured value in solution S3 at values above 0.01 mg per litre (ppm), corresponding to 0.025 µg per g. If the measured values are below these values, report the result as less than 0.01 mg per litre (ppm), corresponding to less than 0.025 µg per g. Additional acceptance criteria for certain metals are provided as follows.

6.2.1.2.10. Polymer Additives

The polymer additive is given in Table 2.

6.2.1.3. Plastic Containers for Ophthalmic Preparations

Plastic containers for ophthalmic preparations are made from plastic composed of a mixture of homologous compounds having a range of molecular weights. Such plastics frequently contain other substances such as residues from the polymerisation process, plasticisers, stabilisers, antioxidants, lubricants and pigments. For deciding the suitability of a plastic for use as a container for ophthalmic preparations, factors such as the composition of the plastic, processing and cleaning procedures, contacting media, adhesives, adsorption and permeability of preservatives, conditions of storage, etc. should be evaluated by appropriate additional specific tests.

Plastic containers for ophthalmic preparations comply with the following tests.

Leakage test; Collapsibility test. Comply with the tests described under Plastic Containers for Parenteral Preparations (6.2.1.1).

Table 2 - Names of polymer additives with teir synonyms

Polymer Additive	Name of Polymer additive as per IUPAC Rules	Synonyms Name
Additive 01	(2*RS*)-2-ethylhexyl benzene-1,2-dicarboxylate	– **di(2-ethylhexyl) phthalate,** – 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester.
Additive 02	zinc (2*RS*)-2-ethylhexanoate	– **zinc octanoate,** – 2-ethylhexanoic acid, zinc salt (2:1), – zinc 2-ethylcaproate.
Additive 03	*N,N2* -ethylenedialcanamide (with *n* and *m* = 14 or 16)	– ***N,N'*-diacylethylenediamines,** – *N,N'*-diacylethylenediamine (in this context acyl means in particular palmitoyl and stearoyl).
Additive 04	Epoxidised Soya Oil	
Additive 05	Epoxidised Linseed Oil	
Additive 06	(EINECS)/Pigment blue 29 (CI77007) ultramarine blue	
Additive 07	2,6-bis(1,1-dimethylethyl)-4-methylphenol	– **butylhydroxytoluene,** – 2,6-bis(1,1-dimethylethyl)-4-methylphenol, – 2,6-di-*tert*-butyl-4-methylphenol
Additive 08	ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4--hydroxyphenyl]butanoate]	– **ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate],** – butanoic acid, 3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]-1,2-ethanediyl ester, – ethylene bis[3,3-bis(3-*tert*-butyl-4-hydroxy-phenyl) butyrate].

cont.

Polymer Additive	Name of Polymer additive as per IUPAC Rules	Synonyms Name
Additive 09	methanetetryltetramethyl tetrakis[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl] propanoate]	– **pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate],** – 2,2-bis[[[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl] propanoyl]oxy]methyl]propane- 1,3-diyl 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoate, – benzenepropanoic acid, 3,5-bis(1,1-dimethyl-ethyl)-4-hydroxy-2,2-bis(hydroxymethyl) propane-1,3-diol ester (4:1), – 2,2-bis(hydroxymethyl)propane-1,3-diol tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate].
Additive 10	4,42 ,43 -[(2,4,6-trimethylbenzene-(1,3,5-triyl)tris methylene)]tris[2,6-bis (1,1-dimethylethyl)phenol]	– **2,22 ,23 ,6,62 ,63 -hexa-*tert*-butyl-4,42 ,43 -[(2, 4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol,** – 1,3,5-tris[3,5-di-*tert*-butyl-4-hydroxybenzyl]-2,4,6-trimethyl-benzene, – phenol, 4,42 ,43 -[(2,4,6-trimethyl-1,3,5-benzenetriyl)tris (methylene)]tris[2,6-bis(1,1-dimethylethyl).
Additive 11	octadecyl 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoate	– **octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate,** – propanoic acid, 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-, octadecyl ester.
Additive 12	tris[2,4-bis(1,1-dimethylethyl)phenyl] phosphite	– **tris(2,4-di-*tert*-butylphenyl) phosphite,** – phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1), – 2,4-bis(1,1-dimethylethyl)phenyl, phosphite.
Additive 13	1,3,5-tris[3,5-bis(1,1-dimethylethyl)-4-hydroxybenzyl]-1,3,5-triazine-2,4,6 (1*H*,3*H*,5*H*)-trione	– **1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione,** – 1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione, 1,3,5-tris[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methyl]-.
Additive 14	3,9-bis(octadecyloxy)-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane	– **2,22 -bis(octadecyloxy)-5,52 -spirobi[1,3,2-dioxaphosphinane],** – 2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane, 3,9-bis(octadecyloxy)-.
Additive 15	1,12 -disulfanediyldioctadecane	– **dioctadecyl disulfide,** – octadecane, 1,12 -dithio-.
Additive 16	didodecyl 3,32 -sulfanediyldipropanoate	– **didodecyl 3,32 -thiodipropionate,** – didodecyl 3,32 -sulfanediyldipropanoate, – propanoic acid, 3,32 -thiobis-, dodecyl diester, – lauryl thiodipropionate.
Additive 17	dioctadecyl 3,32 -sulfanediyldipropanoate	– **dioctadecyl 3,32 -thiodipropionate,** – dioctadecyl 3,32 -sulfanediyldipropanoate, – propanoic acid, 3,32 -thiobis-, octadecyl diester, – stearyl thiodipropionate.
Additive 18	**Component I** 2,4-bis(1,1-dimethylethyl)phenyl biphenyl-4,4′-diyldiphosphonite **Component II** 2,4-bis(1,1-dimethylethyl)phenyl biphenyl-3,4′-diyldiphosphonite **Component III** 2,4-bis(1,1-dimethylethyl)phenyl biphenyl-3,3′-diyldiphosphonite **Component IV** 2,4-bis(1,1-dimethylethyl)phenyl biphenyl-4-ylphosphonite **Component V** 2,4-bis(1,1-dimethylethyl)phenyl phosphate **Component VI** 2,4-bis(1,1-dimethylethyl)phenyl 4′-[bis[2,4-bis(1,1-dimethylethyl)phenoxy] phosphanyl] biphenyl-4-ylphosphonate **Component VII** R-OH:2,4-bis (1,1-dimethylethyl)phenol	

Clarity of aqueous extract; Non-volatile residue. Comply with the tests described under Plastic Containers for Non-Parenteral Preparations (6.2.1.2.).

Eye irritation test. This test is designed to evaluate responses to the instillation of extracts of material under examination in the eye of a rabbit.

Extracting media. (a) Sodium Chloride Injection (b) Vegetable Oil.

Test animals. Select healthy, albino rabbits having no visible eye irritation and not previously used for an eye irritation test. The animal house should be designed and maintained so as to exclude sawdust, wood chips, or other extraneous materials that might produce eye irritation. Examine both eyes of the animals before testing and use only those animals without eye defects or eye irritations.

To test the suitability of the rabbit ocular system in use for a given set of samples, select one test animal and proceed as shown under procedure using 100 µL of a blank prepared as directed under Systemic injection test in one eye and 100 µL of sterile water for injection in the other eye. The rabbit ocular system is suitable if no significant differences are found between the two eyes.

Procedure. Use three albino rabbits for each extract to be examined. Restrain the animals firmly but gently until quiet. Gently pull the lower lid away from the eyeball to form a cup, and instil about 100 µl of *sterile water for injection*. Hold the lid together for about 30 seconds. Instil in to the other eye 100 µl of the sample extract prepared as directed under Systemic injection test. Examine the eyes 24, 48 and 72 hours after instillation. The requirements of the test are met if the sample extract shows no significant irritant response during the observation period over that with the blank extract and the rabbit ocular system is suitable. If irritation is observed in the control eyes treated with sterile water for injection or if the rabbit ocular system is shown not to be suitable, repeat the test using three additional rabbits. In the repeat test, all the rabbits meet the test requirement.

Biological Tests. Perform the test for Biological Reactivity, *In Vitro* (2.2.23). Materials that meet the requirements of this test are not required to undergo testing as described in test for Biological Reactivity, *In Vivo* (2.2.24).

6.2.2. Glass Containers

Glass containers may be colourless or coloured.

Neutral glass is a borosilicate glass containing significant amounts of boric oxide, aluminium oxide, alkali and/or alkaline earth oxides. It has a high hydrolytic resistance and a high thermal shock resistance.

Soda-lime-silica glass is a silica glass containing alkali metal oxides, mainly sodium oxide and alkaline earth oxides, mainly calcium oxide. It has only a moderate hydrolytic resistance.

According to their hydrolytic resistance, glass containers are classified as:

— Type I glass containers which are of neutral glass, with a high hydrolytic resistance, suitable for most preparations whether or not for parenteral use,

— Type II glass containers which are usually of soda-lime-silica glass with high hydrolytic resistance resulting from suitable treatment of the surface. They are suitable for most acidic and neutral, aqueous preparations whether or not for parenteral use,

— Type III glass containers which are usually of soda-lime-silica glass with only moderate hydrolytic resistance. They are generally suitable for non-aqueous preparations for parenteral use, for powders for parenteral use (except for freeze-dried preparations) and for preparations not for parenteral use.

Glass containers intended for parenteral preparations may be ampoules, vials or bottles. The glass used in the manufacture of such containers complies with one of the requirements for hydrolytic resistance given below.

Containers of Type II or Type III glass should be used once only. Containers for human blood and blood components must not be re-used. Glass containers with a hydrolytic resistance higher than that recommended for a particular type of preparation may generally also be used.

Containers for parenteral preparations are made from uncoloured glass except that coloured glass may be used for substances known to be light sensitive; in such cases, the containers should be sufficiently transparent to permit visual inspection of the contents.

Hydrolytic resistance

The tests to be done for defining the type of glass are given in Table 3.

Table 3

Type of container	Test to be done
Type I and Type II glass containers to distinguish from Type III glass containers	Test 1 (surface test)
Type I and Type II glass containers where it is necessary to determine whether the high hydrolytic resistanceis due to the chemical composition or the surface treatment	Tests 1 and 2

Test 1. Carry out the determination on the unused containers. The number of containers to be examined and the volumes of test solution to be used are given in Table 4.

Table 4

Nominal capacity of container (ml)	Number of containers to be used	Volume of test solution to be used for titration (ml)
Up to 3	At least 20	25.0
5 or less	At least 10	50.0
6 to 30	At least 5	50.0
More than 30	At least 3	100.0

Remove any debris or dust from the containers. Rinse each container at least twice with *water* at room temperature. Just before the test rinse each container with freshly prepared *distilled water* and allow to drain. Complete the cleaning procedure from the first rinsing in not less than 20 minutes and not more than 25 minutes. Fill the containers to the brim with freshly prepared distilled water, empty them and determine the average overflow volume.

Heat closed ampoules on a water-bath or in an air-oven at about 50°. Fill the ampoules with freshly prepared *distilled water* to the maximum volume compatible with sealing them by fusion of the glass and seal them. Fill bottles or vials to 90 per cent of their calculated overflow volume and cover them with borosilicate glass dishes or aluminium foil previously rinsed with freshly prepared *distilled water*. Place the containers in an autoclave containing water so that they remain clear of the water. Close the autoclave, displace the air by passage of steam for 10 minutes, raise the temperature from 100° to 121° over 20 minutes, maintain a temperature of 121° for 60 minutes and reduce the temperature from 121° to 100° over 40 minutes, venting to prevent vacuum.

Remove the containers from the autoclave and cool them in a bath of running tap water. Carry out the following titration within 1 hour of removing the containers from the autoclave. Combine the liquids from the containers under examination, measure the volume of test solution specified in Table 2 into a conical flask and add 0.15 ml of *methyl red solution* for each 50.0 ml of liquid. Titrate with *0.01 M hydrochloric acid* taking as the end-point the colourobtained by repeating the operation using the same volume of freshly prepared *distilled water*. The difference between the preparations represents the volume of *0.01 M hydrochloric acid* required by the test solution. Calculate the volume of *0.01M hydrochloric acid* required for each 100.0 ml of test solution, if necessary. The result is not greater than the value stated in Table 5.

Table 5

Capacity of container [corresponding to 90 per cent average overflowvolume (ml)]	Volume of *0.01M hydrochloric* acid /100 ml of test solution	
	Type I or II glass(ml)	Type III glass(ml)
Not more than 1	2.0	20.0
More than 1 but not more than 2	1.8	17.6
More than 2 but not more than 5	1.3	13.2
More than 5 but not more than 10	1.0	10.2
More than 10 but not more than 20	0.80	8.1
More than 20 but not more than 50	0.60	6.1
More than 50 but not more than 100	0.50	4.8
More than 100 but not more than 200	0.40	3.8
More than 200 but not more than 500	0.30	2.9
More than 500	0.20	2.0

Test 2. Examine the number of containers indicated in Table 4. Rinse the containers twice with water and then fill completely with a 4 per cent v/v solution of *hydrofluoric acid* and allow to stand at room temperature for 10 minutes. Empty the containers and rinse carefully five times with water. Carry out the procedure described under Hydrolytic resistance. Compare the results with the limiting values given in Table 4. For Type I glass the values obtained with the hydrofluoric acid-treated containers are closely similar to those stated in the Table for Type I or Type II glass. For Type II glass the values obtained with the hydrofluoric acid-treated containers greatly exceed those given in the Table for Type I or Type II glass and are similar to those given for Type III glass.

Arsenic. Glass containers for aqueous parenteral preparations should comply with the following test. Carry out the test on ampoules the inner and outer surfaces of which are washed five times with freshly distilled *water*.

Prepare a test solution as described in the test for Hydrolytic resistance for an adequate number of containers to produce 50.0 ml. Pipette 10.0 ml of the test solution from the combined contents of all the containers into a flask, add 10.0 ml of *nitric acid* and evaporate to dryness on a water-bath. Dry the residue in an oven at 130° for 30 minutes. Cool, add to the residue 10.0 ml of *hydrazine-molybdate reagent*, swirl to dissolve and heat under reflux on a water-bath for 20 minutes. Cool to room temperature. Determine the absorbance of the resulting solution at the maximum at about 840nm (2.4.7), using 10.0 ml of *hydrazine-molybdate reagent* as the blank. The absorbance of the test solution does not exceed the absorbance obtained by repeating the determination using 0.1 ml of *arsenic standard solution (10 ppm As)* in place of the test solution (0.1 ppm).

6.2.3. Metal Containers

Collapsible Metal Tubes for Ophthalmic Ointments. Metal collapsible tubes comply with the following test for metal particles.

Select a sample of 50 tubes from the lot to be tested and clean each tube by vibration and/or "blowing". (A lot may be either the tube manufacturer's day's production or a consignment delivered to the tube user). Fill the tubes with a suitable molten eye ointment base, close the open end of each tube by a double fold and allow the filled tubes to cool overnight at a temperature of 15° to 20°.

Assemble a metal bacteriological filter with a 4.25-cm filter paper of suitable porosity supported on suitable perforated plate in place of the standard sintered carbon disc and heat it in a suitable manner to a temperature above the melting range of the base. Remove the caps from the cooled tubes and apply uniform pressure to the closed end of each tube in turn, in such a manner that the time taken to express as much of the base as possible through each nozzle is not less than 20 seconds. Collect the extruded base from the 50 tubes in the heated filter, applying suction to the stem of the filter in order to draw the molten base through the filter paper. When the entire melted base has been removed, wash the walls of the filter and the filter paper with three successive quantities, each of 30 ml, of chloroform, allow the filter paper to dry and immediately mount it between glasses for examination.

Examine the filter paper under oblique lighting with the aid of magnifying glass with a graticule of 1mm squares, one of which is sub-divided into 0.2 mm squares and note (a) the number of all metal particles 1mm in length and longer, (b) the number in the range 0.5 mm to less than 1mm and (c) the number in the range 0.2 mm to less than 0.5 mm.

Carry out two further examinations with the filter paper in two different positions so that the lighting comes from different directions and calculate the average number of metal particles counted in each of the three ranges specified. Give each metal particle detected on the filter paper a score as follows and add the scores together.

Particles 1 mm and above	50
Particles 0.5 mm but less than 1 mm	10
Particles 0.2 mm but less than 0.5 mm	2
Particles less than 0.2 mm	Nil

The lot of tubes passes the test if the total score is less than 100 points; if the total score is more than 150 points, the lot fails the test. If the total score is between 100 and 150 (inclusive), the test is repeated on a further sample of 50 tubes and the lot passes the test if the sum of total scores in the two tests is less than 150 points.

6.3. CLOSURES FOR CONTAINERS

A closure for a container for an aqueous parenteral preparation or for a sterile powder is a packaging component which is in direct contact with the drug. A rubber closure is made of materials obtained by vulcanisation (cross-linking) of elastomers with appropriate additives. The elastomers are produced from natural or synthetic substances by polymerization - polyaddition or polycondensation. The nature of the principal components and of the various additives such as vulcanisers, accelerators, stabilising agents, pigments, etc. depends on the properties required for the finished closure. The requirements of this chapter do not apply to closures made from silicone elastomer, to laminated closures or to lacquered closures.

Rubber closures are used in a number of formulations and consequently different closures possess different properties.

*NOTE 8—**Closures will follow the same tests as containers***

Closures made from plastics will be governed by all requirements covered in 6.2.1. and its subsections.

The closures chosen for use with a particular preparation should be such that the components of the preparation in contact with the closure are not adsorbed onto the surface of the closure to an extent sufficient to affect the product adversely. The closure should not yield to the product substances in quantities sufficient to affect its stability or to present a risk of toxicity. The closures should be compatible with the preparation for which they are used throughout the shelf-life of the product.

It shall be the responsibility of the user/manufacturer to ensure the compatibility of the pharmaceutical articles and stability of the product in the package system. Towards this the user / manufacturer shall have in place a system of quality assurance with the supplier / convertor to ensure compliance of the components of the package with the requirement stated in this chapter at all times. The user should take the "risk-based approach" to determine the testing and its frequency required to ensure this.

The following test procedures apply to rubber closures which comprise wads (flat rubber discs), plugs (with or without skirt or flange) and caps (rubber covers held in position on the outsides of the containers by the tension of the rubber) so as to form with their appropriate seals an effective barrier against micro-organisms after sterilisation.

Identification of the type of rubber used for closures is not covered in the following tests. The tests given distinguish elastomer and non-elastomer closures but do not differentiate the various types of rubber.

Description. Rubber closures are elastic and either translucent or opaque; the colour depends on the additives used. They

are homogeneous and practically free from flash and adventitious materials such as fibres, foreign particles and adhering rubber pieces.

Identification

A. Heat 1 g to 2 g of rubber stoppers in a heat resistant test tube over an open flame to dry the sample and continue heating until pyrolysate vapours are condensed near the top edge of the test tube. Deposit a few drops of the condensate on a potassium bromide disc and examine by infrared absorption spectrophotometry (2.4.6), comparing with the spectrum obtained with the type (specimen) sample.

OR

Examine by Attenuated Total Reflectance [ATR] (2.4.6). The spectrum obtained is identical to the spectrum obtained with the type (specimen) sample. If necessary, cut the sample along an appropriate axis, examine the cut surface and compare the spectrum with that obtained with the type (specimen) sample prepared in the similar way.

B. The total ash (2.3.19) is within ±10 per cent of the value obtained with the type sample (specimen).

Preparation of samples. Wash the closures by agitation in a 0.2 per cent w/v solution of an anionic surface-active agent for 5 minutes at room temperature. Rinse five times with *water*, place a number of the washed closures corresponding to a surface area of about 100 cm^2, in a suitable container of borosilicate glass or inert material, and add 200.0 ml of water per 100 cm^2, surface area of the closures and weigh. Cover the mouth of the container with aluminium foil or a borosilicate glass beaker and heat in an autoclave so that a temperature of 119° to 123° is reached within 20 to 30 minutes and maintain at that temperature for 30 minutes. Cool to room temperature over about 30 minutes and make up to the original weight with *water for injection*. Shake and immediately separate the solution from the closures by decantation (Solution A).

Prepare a blank in the same manner using 200.0 ml of *water for injection*.

Dry the treated closures at 64° to 66° at a pressure not exceeding 0.7 kPa for 24 hours.

Appearance of solution. Solution A is not more opalescent than opalescence standard OS3 (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity or alkalinity. To 20.0 ml of solution A add 0.1 ml of *bromothymol blue solution*. Not more than 0.3 ml of *0.01 M sodium hydroxide* or 0.8 ml of *0.01 M hydrochloric acid* is required to change the colour of the solution to blue or yellow respectively.

Light absorption. Carry out the test within 4 hours of preparing solution A. Filter solution A through a membrane filter with a nominal pore size of 0.5µm and reject the first few ml of the filtrate. Measure the light absorption of the filtrate in the range 220 to 360 nm (2.4.7), using as the blank a solution prepared in the same manner as solution A but using 200.0 ml of *water* without the closures. The absorbance is not more than 2.0; if necessary, dilute the filtrate before measurement and correct the results for the dilution.

Reducing substances. Carry out the test within 4 hours of preparing solution A. To 20.0 ml of solution A, add 1 ml of *1 M sulphuric acid* and 20.0 ml of *0.002 M potassium permanganate* and boil for 3 minutes. Cool, add 1 g of *potassium iodide* and titrate immediately with *0.01 M sodium thiosulphate* using 0.25 ml of *starch solution*, added towards the end of the titration, as indicator. Repeat the operation using 20.0 ml of the blank prepared in the test for Light absorption. The difference between the titration volumes is not more than 7.0 ml.

Heavy Metals (2.3.13). 20.0 ml of solution A complies with the limit test for heavy metals, Method A.

Residue on evaporation. Evaporate 50.0 ml of solution A to dryness on a water-bath and dry at 105°. The residue weighs not more than 4.0 mg.

Volatile sulphides. Place closures, cut if necessary, with a total surface area of 20 ± 2 cm^2 in a 100-ml conical flask and add 50.0 ml of a 2 per cent w/v solution of *citric acid*. Place a piece of *lead acetate paper* over the mouth of the flask and maintain the paper in position by placing over it an inverted weighing bottle. Heat in an autoclave at 121±2° for 30 minutes. Any black stain on the paper is not more intense than that of a standard prepared at the same time in the same manner using 0.154 mg of *sodium sulphide* and 50.0 ml of a 2 per cent w/v solution of *citric acid*.

Sterilisation test. The closures 'prepared' in the aforementioned manner shall not soften or become tacky and there shall be no visual change in the closure.

Fragmentation test. This test is applicable to closures intended to be pierced by a hypodermic needle. For closures that are intended to be used for aqueous preparations, place a volume of *water* corresponding to the nominal volume minus 4ml in each of 12 clean vials, close the vials with the 'prepared'closures, secure with a cap and allow to stand for 16 hours. For closures that are intended to be used for dry preparations, close 12 clean vials with the 'prepared' closures. Using a lubricated, long-bevel (bevel angle of 10° to 14°) hypodermic needle with an external diameter of 0.8 mm (21 SWG) fitted to a clean syringe, inject 1 ml of *water* into the vial and remove

1 ml of air; carry out this operation 4 times for each closure, piercing each time at a different site. Use a new needle for each closure and check that the needle is not blunted during

the test. Pass the liquid in the vials through a filter with a nominal pore size of 0.5 µm. Count the number of fragments visible to the naked eye. The total number of fragments is not more than 10 except in the case of butyl rubber closures where the total number of fragments is not more than 15.

Self-sealability. This test is applicable to closures intended to be used with multidose containers. Fill 10 suitable vials with *water* to the nominal volume, close the vials with the 'prepared' closures and secure with a cap. For each closure, use a new hypodermic needle with an external diameter of 0.8mm (21 SWG) and pierce the closure 10 times, piercing each time at a different site. Immerse the vials upright in a 0.1 per cent w/v solution of *methylene blue* and reduce the external pressure by 27 kPa for 10 minutes. Restore the atmospheric pressure and leave the vials immersed for 30 minutes. Rinse the outside of the vials. None of the vials contains any trace of coloured solution.

Biological Tests. Perform the test for Biological Reactivity, *In Vitro* (2.2.23). Materials that meet the requirements of this test are not required to undergo testing as described in test for Biological Reactivity, *In Vivo* (2.2.24).

6.4. Labels on Container

6.4.1. Basic Statutes Governing Labelling

Requirements on Labelling of the packages/container for pharmaceuticals shall be governed as/the following provisions:

- Labelling requirements as/the Drugs & Cosmetics Act, 1940 and the Drugs & Cosmetics Rules, 1945 as amended from time to time.
- Section on Labelling covered in General Notices in the applicable version of the Indian Pharmacopoeia.

7. TABLES

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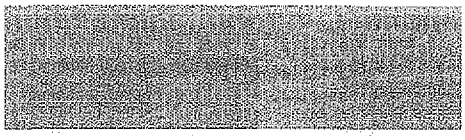
7.1 Names, Symbols and Atomic Weights of Elements

$^{12}C = 12$

Element	*Symbol*	*Atomic Weight*	*Element*	*Symbol*	*Atomic Weight*
Actinium	Ac	(227)	Molybdenum	Mo	95.94
Aluminium	Al	26.9815	Neodymium	Nd	144.24
Americium	Am	(243)	Neon	Ne	20.1797
Antimony	Sb	121.757	Neptunium	Np	(237)
Argon	Ar	39.948	Nickel	Ni	58.6934
Arsenic	As	74.9216	Niobium	Nb	92.9064
Astatine	At	(210)	Nitrogen	N	14.0067
Barium	Ba	137.327	Nobelium	No	(259)
Berkelium	Bk	(247)	Osmium	Os	190.2
Beryllium	Be	9.0122	Oxygen	O	15.9994
Bismuth	Bi	208.9804	Palladium	Pd	106.42
Bohrium	Bh	(264)	Phosphorus	P	30.9738
Boron	B	10.811	Platinum	Pt	195.08
Bromine	Br	79.904	Plutonium	Pu	(244)
Cadmium	Cd	112.411	Polonium	Po	(209)
Caesium	Cs	132.9054	Potassium	K	39.0983
Calcium	Ca	40.078	Praseodymium	Pr	140.9077
Californium	Cf	(251)	Promethium	Pm	(145)
Carbon	C	12.011	Protactinium	Pa	231.03588
Cerium	Ce	140.115	Radium	Ra	(226)
Chlorine	Cl	35.4527	Radon	Rn	(222)
Chromium	Cr	51.9961	Rhenium	Re	186.207
Cobalt	Co	58.9332	Rhodium	Rh	102.9055
Copper	Cu	63.546	Roentgenium	Rg	(272)
Curium	Cm	(247)	Rubidium	Rb	85.4678
Darmstadtium	Ds	(281)	Ruthenium	Ru	101.07
Dubnium	Db	(262)	Rutherfordium	Rf	(261)
Dysprosium	Dy	162.50	Samarium	Sm	150.36
Einsteinium	Es	(252)	Scandium	Sc	44.9559
Erbium	Er	167.26	Seaborgium	Sg	(266)
Europium	Eu	151.965	Selenium	Se	78.96
Fermium	Fm	(257)	Silicon	Si	28.0855
Fluorine	F	18.9984	Silver	Ag	107.8682
Francium	Fr	(223)	Sodium	Na	22.9898
Gadolinium	Gd	157.25	Strontium	Sr	87.62
Gallium	Ga	69.723	Sulphur	S	32.066
Germanium	Ge	72.61	Tantalum	Ta	180.9479
Gold	Au	196.9665	Technetium	Tc	(98)
Hafnium	Hf	178.49	Tellurium	Te	127.60
Hassium	Hs	(277)	Terbium	Tb	158.9253
Helium	He	4.0026	Thallium	Tl	204.3833
Holmium	Ho	164.9303	Thorium	Th	232.0381
Hydrogen	H	1.0079	Thulium	Tm	168.9342
Indium	In	114.82	Tin	Sn	118.70
Iodine	I	126.9045	Titanium	Ti	47.867
Iridium	Ir	192.22	Tungsten	W	183.84
Iron	Fe	55.845	Ununbium	Uub	(285)
Krypton	Kr	83.80	Ununhexium	Uuh	
Lanthanum	La	138.9055	Ununoctium	Uuo	
Lawrencium	Lr	(262)	Ununquadium	Uuq	(289)
Lead	Pb	207.2	Uranium	U	238.0289
Lithium	Li	6.941	Vanadium	V	50.9415
Lutetium	Lu	174.967	Xenon	Xe	131.29
Magnesium	Mg	24.3050	Ytterbium	Yb	173.04
Manganese	Mn	54.9381	Yttrium	Y	88.9059
Meitnerium	Mt	(268)	Zinc	Zn	65.39
Mendelevium	Md	(258)	Zirconium	Zr	91.224
Mercury	Hg	200.59			

The above-mentioned atomic weights are those published in 2001 by the International Union of Pure and Applied Chemistry (*Pure App. Chem.* 2003, 75, 1107).



7.2. Weights and Measure: SI Units

The names and symbols for units of measurement used in this Pharmacopoeia are generally the International System of Units (SI) or the CGS metric units.

The SI comprises three categories of units, namely basic units, derived units and supplementary units. The basic units are given below in Table 1.

The derived units may be formed by combining the basic units according to certain algebraic relationships between the corresponding quantities. Some of these derived units with their special names and symbols and their equivalence with other units are shown in Table 3.

Table 1

Quantity	Name of the basic SI Unit	Symbol
Length	metre	m
Mass	kilogram	Kg
Time	second	s
Electric Current	ampere	A
Thermodynamic temperature	kelvin	K
Amount of Substance	mole	mol
Luminous intensity	candela	cd

The prefixes shown in Table 2 are used to form the names and symbols of the decimal multiples and sub-multiples of SI units.

Table 2

Factor	Prefix	Symbol
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{2}	hecto	h
10^{1}	deca	da
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p

Certain units of the SI which have not yet been classified as basic or derived are known as supplementary units and are shown in Table 4.

Table 4

Quantity	Name of supplementary SI units	Symbol
Plane angle	radian	rad
Solid angle	streradian	sr

Some important and widely used units outside the international system are shown in Table 5.

Table 3

Quantity	Names of derived SI units	Symbol	Expressions in basic SI units	Equivalence with other units
Absorbed dose of ionising radiation	gray	Gy	m^2s^{-2}	1 Gy = 1 joule per kg
Energy, work, quantity of heat	joule	J	$Kg\ m^2s^{-2}$	$1\ J = 10^7$ ergs
Electrical potential, potential difference, electromotive force	volt	V	$Kg\ m^2A^{-1}s^{-3}$	
Electric resistance	ohm	Ω	$Kg\ m^2A^{-2}s^{-3}$	
Force	newton	N	$Kg\ m\ s^{-2}$	$1\ N = 10^5$ dynes
Frequency	hertz	Hz	s^{-1}	1 Hz = 1 cycle per second
Power	watt	W	$Kg\ m^2s^{-3}$	
Pressure	pascal	Pa	$Kg\ m^{-1}s^{-2}$	1 kPa = 7.5 mm Hg = 7.5 torr
Radioactivity	becquerel	Bq	s^{-1}	1 Bq = 2.7036 X 10^{-11} curies

Table 5

Quantity	Unit Name	Symbol	Value in SI units
Time	minute	min	1 min = 60 s
	Hour	h	1 h = 60 min = 3600 s
	day	d	1 d = 24 h = 86400 s
Volume	litre	l	1l d $m^3 = 10^{-3}$ m^3
Mass	tonne	t	1 t = 10^3 kg
Rotational Frequency	revolution per minute	r/min	1 r/min = (1/60) s^{-1}

Table-6 gives the conversion from part per million and Table-7 gives commonly used concentrations and their conversions.

Table-7

µg per ml	mg per ml	g per ml	g per 100 ml (per cent w/w)
1	0.001	0.000001	0.0001
10	0.01	0.00001	0.001
20	0.02	0.00002	0.002
50	0.05	0.00005	0.005
100	0.1	0.0001	0.01
200	0.2	0.0002	0.02
500	0.5	0.0005	0.05
1000	1	0.001	0.1
10000	10	0.01	1

Table-6

Parts per billion (ppb)	Parts per million	µg per g (ppm)	Fraction	Decimal	Per cent w/w
1	0.001	0.001	1/1000000000	0.000000001	0.0000001 per cent
10	0.01	0.01	1/100000000	0.00000001	0.000001 per cent
100	0.1	0.1	1/10000000	0.0000001	0.00001 per cent
1000	1	1	1/1000000	0.000001	0.0001 per cent
5000	5	5	5/1000000	0.000005	0.0005 per cent
10000	10	10	1/100000	0.00001	0.001 per cent
	0	20	2/100000	0.00002	0.002 per cent
	50	50	5/100000	0.00005	0.005 per cent
	100	100	1/10000	0.0001	0.01 per cent
	200	200	2/10000	0.0002	0.02 per cent
	1000	1000	1/1000	0.001	0.1 per cent
	5000	5000	5/1000	0.005	0.5 per cent

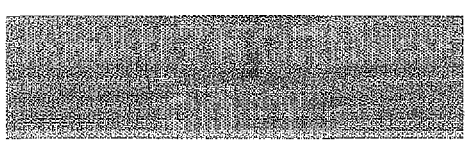
7.3 Abbreviations and Symbols

ALI Annual Limit of Intake

ATCC American Type Culture Collection

$[\alpha]_D^{20}$ Specific optical rotation at 20° at the wavelength of the D line of sodium

$[\alpha]_D^{25}$ Specific optical rotation at 25° at the wavelength of the D line of sodium



bp	Boiling point
BS	British Standard
Ci	Curie
COA	Certificate of Analysis
DNA	Deoxyribonucleic Acid
ED_{50}	Effective Dose 50 (the dose of the preparation that will be effective in 50 per cent of the treated animals)
g	Gram
g	Acceleration due to gravity
HIV	Human Immunodeficiency Virus
ID_{50}	Infective Dose 50 (the dose of the micro-organism that infects 50 per cent of the animals inoculated)
IPRS	Indian Pharmacopoeia Reference Substance
IS	Indian Standard
ISO	International Organisation for Standardization
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry
K	Capacity factor
LD_{50}	Lethal Dose 50 (the dose of the preparation or organism that kills 50 per cent of the animals inoculated.
λ	Wavelength
M	Molarity
MID	Minimum Infective Dose
MLD	Minimum Lethal Dose
mp	Melting point
mEq	Milliequivalent
mmol	Millimole
mol	Gram-molecular weight (mole)
mOsmol	Unit of osmolar concentration expressed as milliosmols of solute
N	Normality
NCIMB	National Collection of Industrial and Marine Bacteria
NCPF	National Collection of Pathogenic Fungi
NCTC	National Collection of Type Cultures
NCYC	National Collection of Yeast Cultures
PD_{50}	Protective Dose 50 (the dose of the preparation that will protect 50 per cent of the animals inoculated)
PDE	Permissible daily exposure
PET	Positron Emission Tomography
ppm	Parts per million
psi	Pounds per square inch
QA	Quality Assurance
QC	Quality Control

R	Resolution
R_f	Used in Thin-layer chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by the solvent front
Rh	Relative humidity
RPs	Radiopharmaceuticals
RS	Reference Substance
RSD	Relative Standard Deviation
RT	Retention Time
rpm	Revolutions per minute
S.D.	Standard Deviation
SN_{50}	Serum neutralising dose 50 (the dose of the preparation that will protect 50 per cent of the cultures against the specified amount of virus)
Sp.gr.	Specific gravity
Wt. per ml.	Weight per millilitre

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